Neurochemical Interactions of Nicotine and Bupropion in the Rat Striatum

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Award date:
2005

Awarding institution:
University of Bath

NEUROCHEMICAL INTERACTIONS OF NICOTINE AND BUPROPION IN THE RAT STRIATUM

Submitted by

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for the degree of PhD of the University of Bath,
Department of Biology & Biochemistry and
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2005.

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ABSTRACT

The rewarding properties of nicotine, which reinforce its self-administration, are related to increased dopamine (DA) release, mediated by nicotinic acetylcholine receptors (nAChRs), in areas of the brain that form the principal terminal fields of the mesolimbic DA system. Currently, bupropion (Zyban®), an atypical antidepressant that has been approved for smoking cessation, inhibits reuptake of DA and noradrenaline (NA). Recently, it has been shown to block nAChRs too. However, the exact mechanisms by which it reduces nicotine intake are still unclear. The present thesis examined the relative importance of these activities, with respect to nicotine-evoked DA release, using various models of increasing complexity.

The effects of bupropion on nicotine-evoked [^{3}H]dopamine ([^{3}H]DA) release were examined in rat striatal synaptosomes and slices using *in vitro* superfusion (Chapter 2). Nomifensine, an established DA transporter (DAT) blocker, was used as a comparison. Bupropion's ability to block DAT and nAChRs appeared to be separated with respect to its concentration. Bupropion (10 μM) reduced nicotine-evoked [^{3}H]DA release in both synaptosomes and slices consistent with its specific inhibition of nicotine-evoked increases in Ca^{2+} fluorescence in SH-SY5Y cells. At the lower concentration of bupropion (0.1 μM) that was proposed to inhibit DAT, an unexpected decrease in nicotine-evoked [^{3}H]DA release was observed in striatal slices. As this effect was reversed by raclopride, a DA D2 receptor antagonist, we postulate that blockade of DAT by bupropion (0.1 μM) results in feedback inhibition via D2 receptors. The effects of a high concentration of nomifensine (10 μM) in slices were consistent with DAT blockade. However, in synaptosomes and SH-SY5Y cells data suggested that nomifensine may also block nAChRs and voltage-operated Ca^{2+} channels (VOCC).

In Chapter 3, the effects of bupropion on nicotine-evoked DA release in the ventral striatum were examined using *in vivo* microdialysis (the construction of a home-made microdialysis system is described in the Appendix), to provide a more physiological model for comparisons with synaptosomes and slices. Behavioural comparisons were made using locomotor activity tests. Systemic administration of bupropion (30 mg/kg) augmented nicotine-evoked DA release and nicotine-induced hyperlocomotion. Its weak DAT blocking actions were inferred to explain these responses. *In vivo*, bupropion failed to exhibit any discernable effect as a nAChR antagonist.

Through a systematic method of analysis using neuronal models of increasing complexity, these studies have demonstrated the intricate effects of bupropion's interactions with DA systems. Indeed better animal models are required to explain its efficacy as a smoking cessation agent. However, it seems that *in vivo*, at least, bupropion is likely to facilitate smoking cessation mainly through its ability to block DAT.

ACKNOWLEDGEMENTS

This PhD has, indubitably, been the most testing "project" that I have undertaken in my academic career and I have many people to thank for their contribution, which has lead to the production of this finished article. However, due to my lack of memory and the shortage of space, I am unable to thank everyone individually, so to everybody who has assisted me in some way, I would like to express my sincere gratitude. Below are some people that I would like to mention as they have been particularly influential:

- My supervisor, Sue "The Boss" Wonnacott, whose continuous encouragement and criticisms reflected by her passion for all things nicotinic made it possible for the completion of this PhD. On a more personal note, her warmth, friendship, guidance and faith in my abilities will never be forgotten. I hope this thesis does it justice...
- My other supervisors, Peter Redfern and David Heal for giving me this invaluable opportunity.
- Helen Rowley, for guiding me through the peaks and troughs of microdialysis and HPLC, and Paul Mitchell for helping me analyse some of my *in vivo* data.
- All the past and present members of the "Nicotinic" and "Worm" Research Labs., for making my time at Bath so enjoyable and exciting. In no special order, thanks to: Adrian Mogg – I hope you're still not doing those South Park impressions, Fred "Mr Cool" Dajas-Bailador – we still have to play that decider set in tennis, Steve Rousseau for his vivacious personality, Chris Sharples – the Ashes have finally come home to England, Ian "early bird" Jones, Jacques Barik for his cleaning frenzies at our house, Virginia Dajas-Bailador, Kate "Missy Pink" Hanrott, Jane Dickinson for "fat chip Thursdays", Steve Plummer, Jenny Oliver, Adrian Wolstenholme, Darran Yates, Adrian "Jolly" Rogers, Kate Ralphs, Sabina, Amy Bradly, Ghazaleh Pashmi, Momna Hejmadi, Marie Toward - my project student, and Sandra.
- All members of 5 West Level 1, in particular: Ewan Basterfield, Louise Anderson, Leslie Moore, Jane, Jean, and Bill. Thanks also to Graham Willmott – my other house-mate, Kevin Smith for his technical expertise on the HPLC system, the workshop engineers at the Dept. of Biology and Biochemistry for making my microdialysis set-ups, and to all the guys in the Pharmacology football team.
- My family in India who, despite being oceans apart, have constantly supported me in all my endeavours.
- Dimple Sidhpura, who is my wife, my friend and my soul mate. Her support has been constant and unselfish and I love her dearly.
- Last and by no means least, my parents, who have sacrificed so much for me and whose unconditional love and support has seen me through many difficult times. I only hope that I can reward your faith with my future success.

This PhD is funded by Abbott Laboratories (previously Knoll Pharmaceuticals).

Dedicated to my late grandparents:

Mr Liladhar Lakhamshi Sidhpura

and

Mrs Zaverben Liladhar Sidhpura

A fact is a simple statement that everyone believes.
It is innocent, unless found guilty.
A hypothesis is a novel suggestion that no one wants to believe.
It is guilty, until found effective.

Edward Teller

PUBLICATIONS AND COMMUNICATIONS

Invited Review

Wonnacott S., Sidhpura N., Balfour D.J., (2005), **Nicotine: from molecular mechanisms to behaviour**, *Current Opinion in Pharmacology*, **5**: 53-59.

In Refereed Journals

Sidhpura N., Redfern P., Wonnacott S., **Comparisons of the effects of bupropion on nicotinic receptor-mediated [^{3}H]dopamine release from rat striatal synaptosomes and slices**, *Molecular Pharmacology*, (in preparation).

Sidhpura N., Rowley H.L., Heal D.J., Redfern P., Wonnacott S., **Bupropion promotes dopamine release and locomotor activation *in vivo* without inhibiting nicotinic acetylcholine receptor responses**, *Neuropsychopharmacology*, (in preparation).

Communications

Sidhpura N., Rowley H.L., Heal D.J., Mitchell P.J., Redfern P., Wonnacott S., (2004), **Neurochemical interactions of nicotine and bupropion in the rat striatum**, *British Association for Psychopharmacology*, Harrogate, UK.

Sidhpura N., Rowley H.L., Heal D.J., Mitchell P.J., Redfern P., Wonnacott S., (2003), **Actions of bupropion on nicotine-evoked dopamine release in the rat striatum**, *Society for Research on Nicotine and Tobacco*, Padova, Italy.

Sidhpura N., Redfern P., Wonnacott S., (2003), **Actions of bupropion on nicotine-evoked dopamine release in the rat striatum**, *Society for Neuroscience Abstracts*, 322.3.

Sidhpura N., Kenyon R., Redfern P., Wonnacott S., (2003), **Actions of bupropion on nicotine-evoked dopamine release in the rat striatum**, *1st UK Nicotinic Receptor Club Meeting*, Lilly Research Centre, Windlesham, UK and *Behavioural Pharmacology*, **13**: 503-504.

Oral Presentation

Sidhpura N., (2004), **Neurochemical interactions of nicotine and bupropion in the rat striatum**, *British Pharmacological Society*, Bath, UK.

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ABBREVIATIONS

$[^3H]DA$	$[^3H]$dopamine
$[Ca^{2+}]_i$	intracellular calcium ion concentration
5-HT	5-hydroxytryptamine
$^{86}Rb^+$	rubidium ion
α-Bgt	α-bungarotoxin
ACh	acetylcholine
AChE	acetylcholinesterase
aCSF	artificial cerebrospinal fluid
αCtxMII	α-conotoxin MII
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
AP-V	DL-2-amino-5-phosphopentanoic acid
AUC	area under the concentration curve
BBB	blood-brain barrier
BSA	bovine serum albumin
CB1	cannabinoid-1
C_{max}	peak concentration
CNS	central nervous system
cpm	counts per minute
CYP	cytochrome P450
DA	dopamine
DAT	dopamine transporter
DHβE	dihydro-β-erythroidine
DNQX	6, 7-dinitroquinoxaline-2,3-dione
EC_{50}	agonist concentration which evokes a half-maximal response
ECD	electrochemical detection
EDTA	ethanediamino-N,N'-tetraacetic acid
GABA	γ-aminobutyric acid
GP	globus pallidus
HPLC	high pressure liquid chromatography
HPLC-ECD	high pressure liquid chromatography with electrochemical detection
I.D.	internal diameter
i.p.	intraperitoneal
IC_{50}	antagonist concentration which inhibits a half-maximal response
ICSS	intracranial self-stimulation
ID_{50}	antagonist dose which inhibits a half-maximal response
IT	intratelecephalically projecting
K_d	dissociation constant
K_i	inhibition constant
LDT	laterodorsal tegmental nucleus

LTP	long-term potentiation
MAO	monoamine oxidse
mGluR	metabotropic glutamate receptor
MLA	methylcaconitine
NA	noradrenaline
NAc	nucleus accumbens
NAc_{core}	core subregion of the nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NAc_{shell}	shell subregion of the nucleus accumbens
n_H	Hill number
NMDA	N-methyl-D-aspartate
NRT	nicotine replacement therapy
O.D.	outer diameter
ODS	octa decyl silane
PFC	prefrontal cortex
PT	pyramidal tract
s.c.	subcutaneous
S.E.M.	standard error of mean
SN	substantia nigra
SNc	substantia nigra pars compacta
SSRI	selective serotonin reuptake inhibitor
STN	subthalamic nucleus
$t_{½}$	half-life
$t_{½}d$	distribution half-life
$t_{½}e$	elimination half-life
TCA	tricyclic antidepressant
t_{max}	time to reach peak concentration
TPP	tegmental pedunculopontine nucleus
TSS	Tyrode's salt solution
TTX	tetrodotoxin
UV	ultraviolet
VOCC	voltage-operated calcium channels
VP	ventral pallidum
VTA	ventral tegmental area
WHO	World Health Organisation

The nomenclature for all nicotinic acetylcholine receptor subunits described in this thesis is based on the NC-IUPHAR subcommittee recommended definitions published in 1999 (Lukas et al., 1999).

CHAPTER 1

General Introduction

1.1 Drug addiction

Drug addiction is a chronically relapsing disorder characterised by a compulsion to seek and take a drug coupled with the inability to control drug intake despite serious negative consequences (Koob et al., 2004). Such activities often involve the development of repetitive and nearly automatic behaviours that we call habits. Drug addiction is a worldwide public-health crisis, and exerts corrosive effects at family and societal levels, which include health care expenditure, lost earning, and costs associated with crime and accidents. The World Health Organisation (WHO) estimated that there are 2 billion alcohol users, 1.3 billion tobacco users, and 185 million users of illicit drugs (The WHO Report, 1999). In 2001, these three categories together contributed to 12.4% of deaths worldwide. In the United States alone, approximately 590000 deaths are caused by addictive drugs (NIDA website, 2005). The costs of drug abuse are an enormous burden that affects all society – those who abuse these substances, and those who do not (Fig. 1.1).

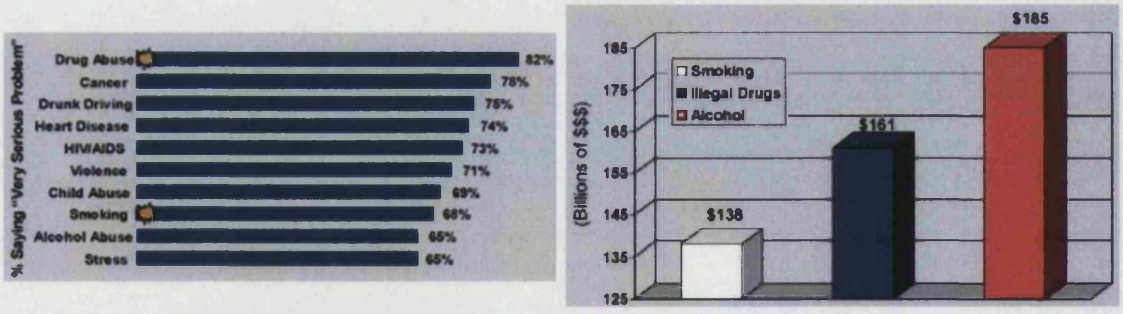


Fig. 1.1 Epidemiology of major health problems and approximate costs to US society for substance abuse in the USA.
(source: www.drugabuse.gov)

The likelihood of developing addiction is a complex function of many factors, including the individual's personality and other psychological factors (social adequacy), background (family, neighbourhood, school and peer factors), substance availability, an individual's genotype, and the pharmacological properties of the drug (Fig. 1.2). Interactions between genes and environmental factors with genes also are likely to be important (van den Bree, 2005). When addictive drugs are used repeatedly by vulnerable humans, molecular changes in the brain promote continued drug taking that becomes increasingly difficult for the individual to control. Once addiction has taken hold, it tends to

follow a chronic course, in which periods of abstinence are followed by relapse to active drug use (Hyman and Malenka, 2001).

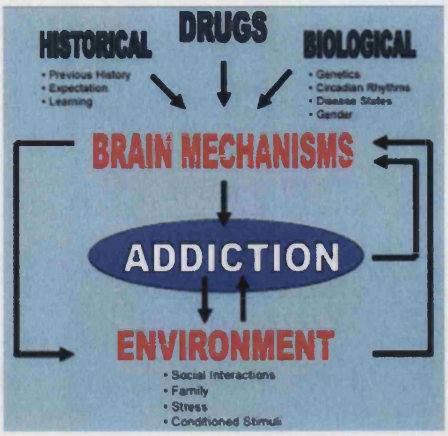


Fig. 1.2 Schematic of various factors involved in addiction. (source:www.drugabuse.gov)

Addictive drugs, such as cocaine and amphetamine, are both rewarding and reinforcing. A reinforcer can be defined operationally as any event that increases the probability of a response. Reward is often defined similarly but with some positive affective colouring, such as pleasure or hedonia (Koob, 1992). Several sources of reinforcement may contribute to addiction. In positive reinforcement, a rewarding stimulus increases the probability of any behaviour that results in a drug being administered, and so is critical for establishing addictive behaviour. In negative reinforcement, the incentive for drug administration is relief of a painful or unpleasant state (Roberts and Koob, 1997). Both positive and negative reinforcement are important for maintaining drug use following the development of addiction.

"Drug dependence" is the clinical term favoured by the Diagnostic and Statistical Manual of Mental Disorders (fourth edition). However, "dependence" and "addiction" are not scientifically equivalent. Dependence refers to an adapted state of cells, circuits or organ systems that occurs in response to excessive drug stimulation, such that when unmasked by drug cessation, this adapted state can result in the production of cognitive, emotional or physical withdrawal symptoms (Hyman and Malenka, 2001). In addition to dependence, addictive drugs can produce sensitisation or tolerance, as well as withdrawal. Sensitisation is an increased response to a drug effect following repeated administration of the drug (Di Chiara, 1998). A recent conceptualisation of the role of sensitisation in drug addiction posits that a motivational state described as "wanting" is progressively increased by repeated exposure to drugs of abuse (Robinson and Berridge, 1993). Conversely, tolerance is defined as a decrease in drug-induced effect despite a constant dose, or a need for increasing dosage to maintain a stable effect. Both sensitisation and tolerance to the stimulant effects of drugs of abuse are the result of changes in an individual's sensitivity to the drugs themselves. These changes are referred to as neuroadaptations. Counteradaptation refers to processes that are initiated to oppose the effects of drugs of abuse. Withdrawal is a counteradaptive process, which describes a state of adverse effects, both physical and psychological, that occurs after cessation of drug intake (Rang et al., 2003).

The universal hallmark of addiction is craving (psychological dependence) whereby addicts experience an intense desire to reinstate drug use. Drug craving and

relapse are precipitated by re-exposure to a previously abused drug, an environment associated with prior drug use, drug paraphernalia or a stressful life event. Such encounters are examples of "cues" that are "conditioned" with the experience of drug-taking. Cues associated with either the rewarding effects of drug taking (conditioned positive reinforcement) or the negative effects of drug withdrawal (conditioned negative reinforcement) are two major psychological factors that contribute to drug dependence (Fig. 1.3).

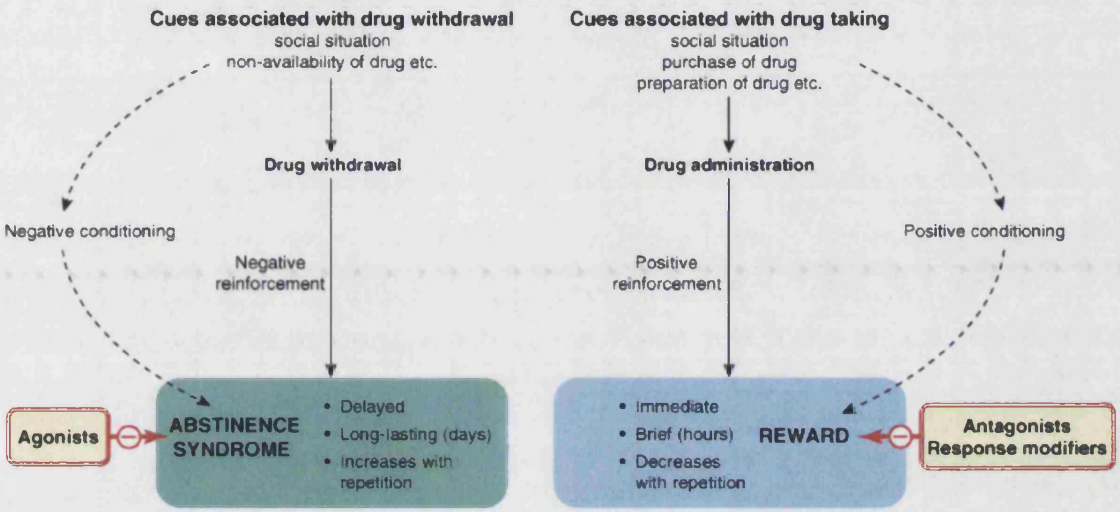


Fig. 1.3 Schematic of psychological factors involved in drug dependence.
(source: Rang et al., 2003)

Many neurotransmitters, including γ-aminobutyric acid (GABA), glutamate, acetylcholine (ACh), DA, serotonin and endogenous opioid peptides, have been implicated in the effects of various types of drugs of abuse (Volkow and Li, 2004). Of these, DA has been consistently associated with the reinforcing effects of most drugs of abuse (Di Chiara, 1999). Like natural reinforcers such as food, water and sex, virtually all drugs of abuse increase extracellular DA concentrations in the mesolimbic DA pathway (see section 1.2.2). Drugs such as cocaine, amphetamine, methamphetamine and ecstasy increase extracellular DA by inhibiting its reuptake or promoting DA release through their effects on DAT (Madras et al., 1989). Other drugs, such as nicotine, alcohol, opiates and marijuana, work indirectly by stimulating neurones (GABA-mediated or glutamatergic) that modulate DA cell firing through their effects on nicotine, GABA, μ-opiate or cannabinoid CB1 receptors, respectively (Kreek et al., 2002). However, nicotine can also have direct effects on DA release by stimulating nAChRs on DA neurones (discussed in section 1.4).

If natural reinforcers increase DA, why do they not lead to addiction? The difference lies in the qualitative and quantitative differences in DA increases induced by addictive drugs, which are greater in magnitude (at least five- to ten-fold) and duration

than those induced by natural reinforcers (Wise, 2002). Furthermore, DA increases produced by natural reinforcers undergo habituation upon repeated exposure, whilst those induced by drugs of abuse do not (Di Chiara, 2002), i.e. non-decremental stimulation of DA transmission from repeated drug use strengthens the motivational properties of the drug, which does not occur for natural reinforcers (Volkow and Li, 2004).

1.2 Dopaminergic pathways in the brain relevant to addiction

Dopaminergic neurones form a number of pathways in the central nervous system (CNS). The two major systems are the nigrostriatal (see section 1.2.1) and mesocorticolimbic system (see section 1.2.2).

1.2.1 The nigrostriatal pathway

The nigrostriatal pathway accounts for about 75% of brain DA and consists of cell bodies in the substantia nigra (SN), in particular the pars compacta (SNc), with axons that terminate in the striatum. These fibres ascend via the medial forebrain bundle and then the internal capsule to innervate the caudate nucleus, the putamen (the caudate and putamen are fused in rodents, but not in primates and humans), and the globus pallidus (GP; Fig. 1.4). The caudate-putamen in rodents is commonly known as the dorsal striatum. The nigrostriatal pathway is vitally important in motor control, and degeneration of this system is the key feature of Parkinson's disease (Quik, 2004). In rats, there are an estimated total of 20000 DA cell bodies in the two SN. A single axon from one of these DA cells branches substantially to give rise to thousands of synaptic boutons in the striatum. Many of these synaptic connections are made with medium-sized spiny neurones that form the output pathways of the striatum (see section 1.4.5.2).

1.2.2 The mesocorticolimbic pathway

The mesolimbic and mesocortical dopaminergic systems (Fig. 1.4) arise from dopaminergic cells located in the ventral tegmental area (VTA). The dopaminergic cells of the VTA and SNc form a continuous layer and project to adjacent and overlapping terminal fields. Hence the boundaries between these "systems" are not well defined (Bressan and Crippa, 2005). DA cells of the VTA project most strongly to the nucleus accumbens (NAc; also known as the ventral striatum) and olfactory tubercule, but also

innervate the septum, amygdala and hippocampus. This subset of projections is known as the mesolimbic dopaminergic pathway and is thought to be important for motivational function. DA cells in the medial VTA that project to the medial prefrontal, cingulate and perirhinal cortex are known as the mesocortical dopaminergic pathway and are involved in modulating attention and other cognitive processes. Due to the overlap between the mesolimbic and mesocortical DA neurones, the two systems are often referred to as the mesocorticolimbic pathway (Wise, 2004).

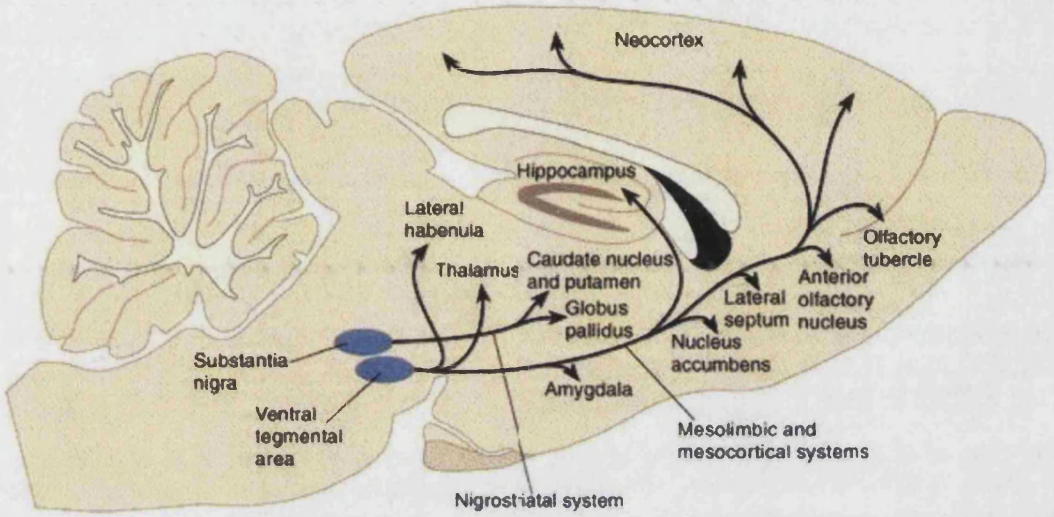


Fig. 1.4 Sagital section through the rat brain depicting the nigrostriatal and mesocorticolimbic dopaminergic pathways.
(source: www-unix.oit.umass.edu)

The role of the mesocorticolimbic DA system in reward related functions originates from the findings of Olds and Milner in 1954. When electrodes were placed in discrete areas of the brain, they observed that rats would actively self-stimulate these areas, often to the exclusion of all other activities, including eating. The circuits involved in this process have been referred to as the reward system. Since then, much focus has been on the mesolimbic dopaminergic pathway, as several studies using animal models of reward, and by measuring *in vivo* DA release with microdialysis, reveal that natural rewarding stimuli such as food, water and sex, and drugs of abuse such as cocaine, amphetamine and nicotine, increase DA release in the NAc (Di Chiara, 1999; Bressan and Crippa, 2005).

The link to nicotine addiction was obtained from rodent self-administration studies, which revealed that nicotine self-administration was abolished by lesions of the mesolimbic dopaminergic pathway, and also by DA receptor antagonists (Corrigall and Coen, 1991; Corrigall et al., 1992). Since nicotine evoked DA release from the so-called reward pathways, the interpretation was made that this system may have a pivotal role in the development and maintenance of tobacco addiction in humans.

1.3 Nicotine addiction

Cigarette smoking has been the most popular method of taking nicotine since the beginning of the 20th century. In 1989, the U.S. Surgeon General issued a report that concluded that cigarettes and other forms of tobacco, such as cigars, pipe tobacco, and chewing tobacco, are addictive and that nicotine is the drug in tobacco that causes addiction. Smoking is the third leading cause of death in the United States, resulting in approximately 440000 premature deaths each year with an annual expenditure of more than $75 billion in direct medical costs (NIDA Info Facts, 2005). WHO epidemiologists predict that unless there is a dramatic change in present trends, tobacco will be killing 8.4 million people a year by the late 2020s (Fig. 1.5; The WHO Report, 1999).

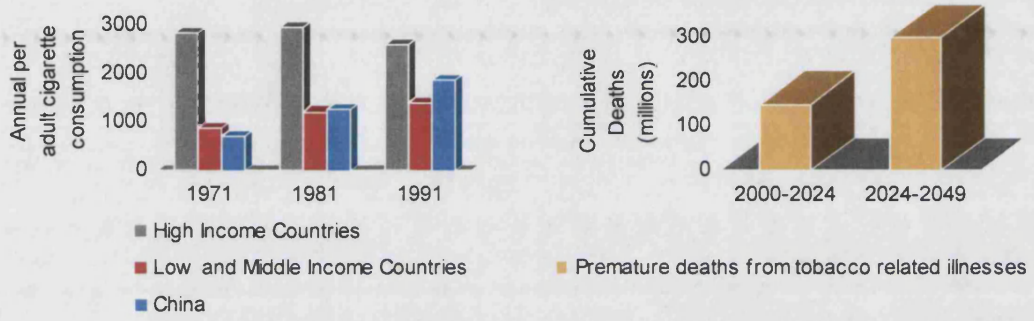


Fig. 1.5 Epidemiology for the growth of cigarette consumption and projected deaths from tobacco related illnesses.
(source: The World Health Report, 1999)

1.3.1 The history of nicotine

Tobacco is a native plant of the American continent. Historians believe tobacco began growing in the Americas around 6000 BC, and that American Indians started the use of tobacco as early as the 1st century BC for hedonistic, medicinal and ceremonial purposes. These effects were later traced to the actions of nicotine, which is one of many cholinomimetics that have been used for these purposes. By the time Europeans arrived on the American continent in the 15th century, smoking of tobacco among indigenous American people was widespread and native tribes were not only growing and consuming tobacco but also trading tobacco leaves. Smoking spread to Europe during the 16th century, coming to England mainly as a result of its enthusiastic espousal by Sir Walter Raleigh at the court of Queen Elizabeth I. It was then that tobacco plants were given the name *Nicotiana tabacum* (Fig. 1.6) in honour of Jean Nicot (1530-1600), who had introduced tobacco chewing to Catherine de Medici of France (1560) and promoted the

importation and cultivation of the plant. However, King James I strongly disapproved of tobacco and initiated the first antismoking campaign in the early 17th century. The first suggestion that tobacco smoking might be addictive was reported in 1610 when Sir Francis Bacon noted that trying to quit the habit was very difficult (Leavey, 1998).

Fig. 1.6 A field of tobacco plants in Cuba.
Nicotiana tabacum is a hardy plant and so is grown in many parts of the world, with the two largest exporters being the USA and Brazil.

Commercial cultivation of tobacco in America began in the early 17th century, and the first successful shipload was sent to England in 1616. By the late 1620s, an estimated 500000 lbs of tobacco was being brought into London every year. Tobacco was now common among all sectors of society and people learnt to smoke, usually by sharing a communal pipe. In 1730, the first American tobacco factories were constructed producing pipe tobacco, cigars and snuff. By the end of the 18th century, snuff-taking was in decline and a revival in smoking had begun in the form of cigars. However, it was the invention of the manufactured cigarette that transformed tobacco smoking into a truly mass habit. James Duke was the first tobacco entrepreneur to use cigarette-making machines. Cigarette smoking among men increased during World War I when cigarettes were included in soldiers' rations. It remained socially unacceptable for women to smoke until the Suffragette movement in the 1920s, when significant numbers of women started to smoke (Leavey, 1998). Currently, cigarettes account for more than 90% of tobacco consumption and nicotine has become far better known than most other cholinotropic substances as it confers an interoceptive "pleasurable effect" (Di Chiara and Imperato, 1988) or euphoria that has instigated and sustained tobacco use by large proportions of human populations virtually the world over.

1.3.2 What is nicotine?

Nicotine, the principal psychoactive agent in tobacco, is one of 4000 chemicals found in the smoke of tobacco products such as cigarettes, cigars and pipes. Nicotine is a colourless liquid

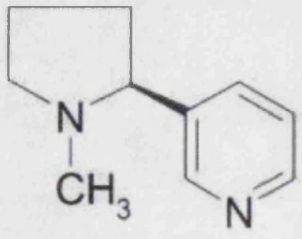


Fig. 1.7 The chemical structure of (-)nicotine.
Nicotine is a water and lipid soluble drug which, in the free base form, is readily absorbed via respiratory tissue, skin, and gastrointestinal tract. At pH 7.4, about 70% of it is ionised in the form shown.

that turns brown when burned and acquires the odour of tobacco when exposed to air. Nicotine is a tertiary amine consisting of a pyridine and a pyrrolidine ring (Fig. 1.7).

1.3.2.1 Pharmacokinetics of nicotine

Nicotine absorption can vary, partly by the method in which it is consumed. Therefore, the time taken for it to reach the brain is a major factor that governs its addictive properties. An average cigarette contains about 0.8 g of tobacco and 9-17 mg nicotine, of which approximately 10% is normally absorbed by the smoker (Rang et al., 2003). Nicotine, absorbed from cigarettes through the lungs and then via arterial blood, reaches the brain in about seven seconds, almost twice as fast as when the drug is administered intravenously (Benowitz, 1996). Absorption of nicotine is pH dependent. At an acid pH of smoke from cigarettes (pH 5.5), nicotine is mostly ionised and does not readily penetrate cell membranes. At the alveoli of the lungs, nicotine is buffered to physiological pH and rapidly crosses cell membranes (Salin-Pascual et al., 2003). Nicotine from smokeless tobacco (snuff and chewing tobacco) is absorbed through the oral mucosa. Absorption here is considerably slower than that from inhaled cigarette smoke, resulting in a later and long-lasting peak in plasma nicotine concentration (Fig. 1.8).

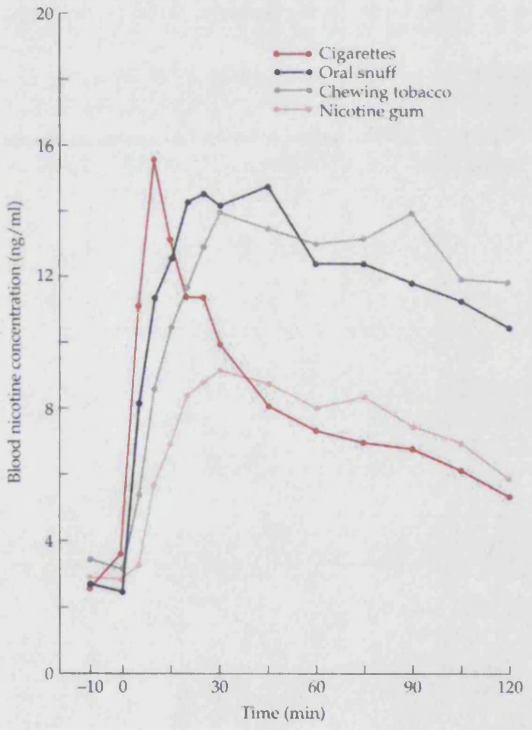


Fig. 1.8 Nicotine concentrations in plasma during smoking.
Blood concentrations during and after cigarette smoking (1⅓ cigarettes) for 9 min and use of oral snuff (2.5 g), chewing tobacco (average 7.9 g), and nicotine gum (two 2 mg pieces) for 30 min. The points represent average values for 10 subjects (adapted from Feldman et al., 1997).

Nicotine concentrations in a cigarette smoker's (on average 20 cigarettes a day) plasma during the day typically range from 20-40 ng/ml. Arteriovenous differences during cigarette smoking are substantial, with arterial levels exceeding venous levels six- to ten-fold (Benowitz, 1996). Absorption of nicotine from the gum is gradual, with plasma levels of nicotine much lower than those achieved after cigarette smoking. Frequent dosing and a correct chewing technique are necessary to achieve good nicotine absorption from the oral mucosa (Tutka et al., 2005). Nicotine from the transdermal patch is slowly absorbed. Plasma concentrations rise gradually over 6-10 h,

reaching a plateau over the next 8-12 h, followed by a slow decline over the final 6 h. Nicotine delivered from the nasal spray is absorbed through the nasal mucosa. The absorption is very rapid and peak arterial plasma levels are reached in about 5 min after administration (Salin-Pascual et al., 2003).

Following absorption, nicotine readily reaches many organs and tissues and undergoes extensive metabolism. The initial distribution half-life ($t_{½}d$) of nicotine in blood is estimated to be 4-10 min, and the elimination half-life ($t_{½}e$) is approximately 2 h. Repeated exposure to nicotine produces a steady state for about 6-8 h. However, the terminal half-life of nicotine is 20 h or more, presumably due to the slow release of nicotine from various bodily tissues (Benowitz, 1996). After acute intra-arterial or intravenous administration of nicotine in rats, mean $t_{½}d$ and $t_{½}e$ of nicotine are approximately 10 and 90 min, respectively. Chronic subcutaneous nicotine injections yield a global half-life of 44-49 min (Mathieu-Kia et al., 2002). Pharmacokinetic studies in rats have shown that levels of nicotine in the brain averages about two or three times higher than that in the blood (Rowell and Li, 1997). Whether a similar situation occurs in humans is unknown at present.

About 80-90% of nicotine is metabolised in the liver, with small amounts in the kidneys, lungs and brain. About 70-80% of nicotine is metabolised to cotinine via C-oxidation, and another 4% to nicotine N′-oxide (Fig. 1.9; Salin-Pascual et al., 2003).

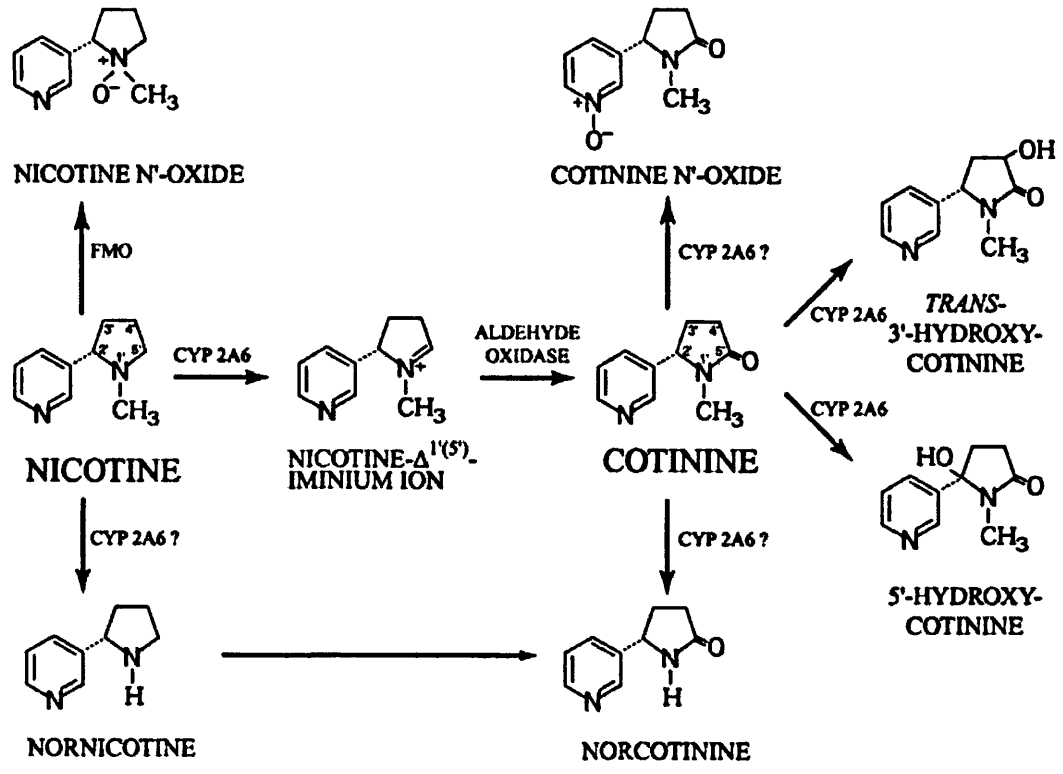


Fig. 1.9 The major pathways of nicotine metabolism in humans.
CYP, cytochrome P450; FMO, flavin mono-oxygenase (adapted from Tutka et al., 2005)

Cotinine is the most abundant metabolite found in urine. Several cytochrome P450 (CYP) enzymes, as well as flavin mono-oxygenases, are purported to play a role in nicotine metabolism, but CYP 2A6 and aldehyde oxidase are the principal enzymes involved in converting nicotine to cotinine. Cotinine has a much longer half-life than nicotine (14-20 h) and consequently is used as a marker for nicotine intake (Salin-Pascual et al., 2003). The main organ for nicotine excretion is the kidney. Renal clearance of nicotine, which depends on urine pH, accounts for 2-35% of total nicotine clearance. Some nicotine is also excreted in the saliva (Tutka et al., 2005).

1.3.3 How does nicotine deliver its effects?

Nicotine can act both as a stimulant and a sedative. Immediately after exposure to nicotine, there is a "kick" caused by discharge of adrenaline from the adrenal cortex. This stimulates the CNS and other endocrine glands causing a sudden release of glucose as well as an increase in blood pressure, respiration and heart rate. In addition, nicotine causes the release of DA in the brain regions that control pleasure and motivation (see section 1.4.6 for nicotinic modulation of DA release). Nicotine can also exert a sedative effect, depending on the level of the smoker's nervous system arousal and the dose of nicotine taken (NIDA Research Report, 1998).

Repeated exposure to nicotine results in the development of tolerance, whilst cessation is followed by a withdrawal syndrome that may last from a few weeks to several months (Table 1). Smokers, however, can regulate nicotine intake by self-titration to avoid the negative psychological effects of high nicotine levels, whilst preventing symptoms of withdrawal. Thus,

PSYCHOLOGICAL	PHYSIOLOGICAL
Anger	Constipation
Anxiety	Dizziness
Confusion	Dry mouth
Depression	Headache
Fearfulness	Hunger
Hostility	Mouth ulcers
Impatience	Pain in limbs
Irritability	Sleep disturbances
Nicotine craving	Sore throat, gums or tongue
Panic	Sweating
Poor concentration	
Restlessness	

Table 1 Possible symptoms as a result of nicotine withdrawal

after termination of each cigarette, blood levels of nicotine rapidly peak and fall. In addition to this intermittent spike pattern, the overall levels of nicotine across the daylight period gradually increase in the morning, reaching maximal levels during the afternoon and late evening, and finally fall during the night (Mathieu-Kia et al., 2002). Smokers achieve this by manipulating nicotine intake through the numbers of puffs per cigarette, the intensity of puffing, the depth of inhalation, and changing the puff volume. As tolerance to nicotine is lost overnight, smokers often report that the first cigarettes of the day are the strongest and/or the "best".

A smoker's chronic exposure to nicotine can result in addiction. Part of the reason lies in the rapid transit time of nicotine to the brain coupled with its relatively short half-life, thus allowing smokers to regulate their nicotine intake throughout the day. However, paired associations between nicotine intake (e.g. cigarette puffs) and environmental situations (e.g. going to the pub or a party) are more pertinent for its addictive nature. As nicotine dependence develops, previously neutral environmental stimuli that are associated repeatedly with episodes of smoking behaviour acquire the ability, through Pavlovian-conditioning processes, to elicit brain activation and craving for tobacco in dependent subjects. Without associated environmental influences, nicotine appears to be relatively weak in animal models of reinforcement (Le Foll and Goldberg, 2005).

The ability of nicotine to activate the mesolimbic DA system by stimulating nAChRs is also a principal component underlying its addictive characteristics (Corrigall et al., 1992).

1.4 Nicotinic acetylcholine receptors

The nAChR belongs to a superfamily of ligand-gated ion channels, which include the 5-hydroxytryptamine (5-HT) type 3, GABA type A and C, and glycine receptors (Changeux et al., 1998; Picciotto et al., 2001; Karlin, 2002; Hogg et al., 2003; Gotti and Clementi, 2004; Jensen et al., 2005). These receptors are known as Cys-loop receptors, as all of them have a conserved sequence containing a pair of cysteines separated by 13 residues and linked by a disulphide bridge (Hogg et al., 2003; Unwin, 2005). These receptors are the target for pharmacologically administered nicotine, and normally respond to endogenous ACh in the muscle, autonomic ganglia and CNS. Muscle nAChRs are confined to the skeletal neuromuscular junction where they mediate neuromuscular transmission; ganglionic nAChRs are responsible for mediating transmission at sympathetic and parasympathetic ganglia; CNS nAChRs are widespread in the brain and are considered to have a modulatory role on neurotransmitter release (Role and Berg, 1996; Wonnacott, 1997). Both ganglionic- and CNS-type nAChRs are collectively known as neuronal nAChRs.

1.4.1 Structure

Early studies to elucidate the subunit composition and structure of nAChRs were performed on receptors isolated from the organs of the electric ray, *Torpedo californica*, and electric eel, *Electrophorus electricus*, as these tissues were rich in nAChRs (Fig. 1.10).

Fig. 1.10 Photographs of the electric ray, *Torpedo californica*, and electric eel, *Electrophorus electricus*.

The nAChR is a 290 kDa, hetero-pentameric glycoprotein assembled from a ring of five homologous membrane-spanning subunits (α, α, β, γ or ε, δ). The subunits have in common a large extracellular N-terminal ligand-binding domain, four predicted transmembrane regions (M1-M4 in each subunit), and an extended cytoplasmic loop giving it a total length normal to the membrane plane of about 170 Å (Fig. 1.11a; Unwin, 2003; Unwin, 2005). This pseudo 5-fold arrangement delineates a cation-selective pathway across the membrane pore when the channel is open, but a robust barrier to ions when it is closed (Fig. 1.11b). The ligand-binding domain shapes a long, approximately 20 Å diameter central vestibule and has two binding sites for ACh (αγ and αδ), which are about 40 Å from the membrane surface on opposite sides of the pore (Unwin et al., 2002). The pore makes a narrow water-filled path across the membrane and contains the gate, which opens when ACh occupies both binding sites. The intracellular domain shapes another, small vestibule, having narrow lateral openings for the ions (Karlin, 2002; Unwin, 2005).

1.4.1.1 Ligand-binding domain

The receptor subunits in the ligand-binding domain are each, presumed to be, organised around two sets of β-sheets packed into a curled β-sandwich and joined through a disulphide bridge forming the Cys loop, as shown by the structure of the closely related soluble protein, ACh-binding-protein (Fig. 1.12a; Brejc et al., 2001). The two ACh-binding sites, which lie at the α-γ and α-δ subunit interfaces (Fig. 1.12b), are each formed

of six loops, termed A-F. The α-subunit, which harbours the principal component of the binding-site, comprises the A, B and C loops, while the complementary component of the

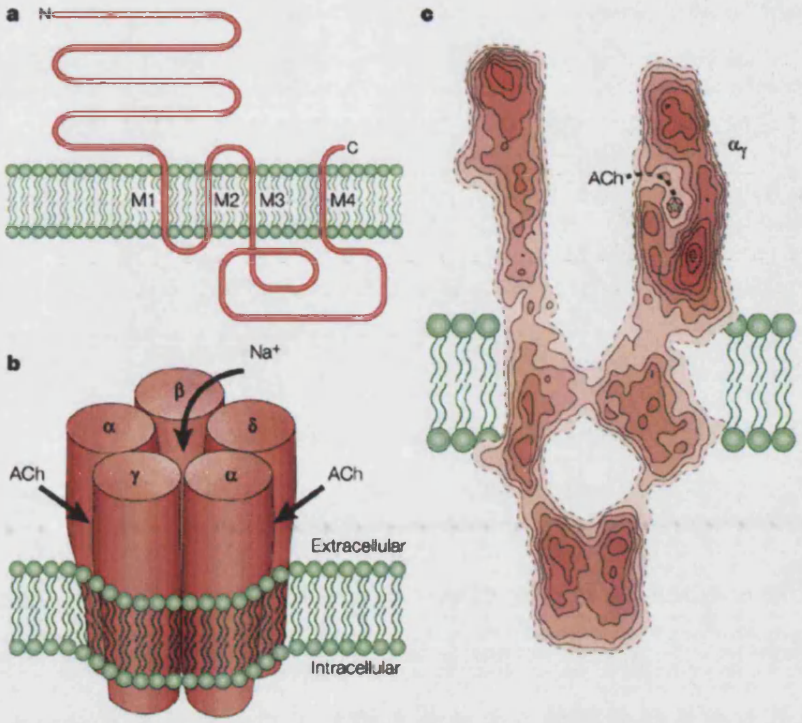


Fig. 1.11 Structure of the nAChR.
(a) Schematic demonstrating the transmembrane topology of a single nAChR subunit. The four membrane spanning domains (M1-M4) are separated by extracellular and intracellular loops of varying lengths. The large extracellular hydrophilic N-terminal region (200 amino acids) forms the ligand-binding domain, possessing sites for agonists, antagonists, ions and other modulators. Three closely spaced hydrophobic sequences form the M1-M3 transmembrane domains. A large intracellular hydrophilic region (100-200 amino acids) that is variable in sequence links the M3 and M4 domains. It offers several putative phosphorylation sites for intracellular ligands that can modulate and regulate the nAChR.
(b) Schematic representation of the quaternary structure, showing the arrangement of the subunits in the muscle-type receptor, the location of the two ACh-binding sites (between an α- and a γ-subunit, and an α- and a δ-subunit), and the axial cation-conducting channel.
(c) A cross-section through the 4.6 Å structure of the nAChR determined by electron microscopy of tubular crystals of *Torpedo* membrane embedded in ice. Dashed line indicates the proposed path to binding site.
(adapted from Miyazawa et al., 1999; Karlin, 2002).

adjacent subunit comprises the D, E and F loops (Fig. 1.12c; Brejc et al., 2001; Changeux and Edelstein, 2001). Modelling of the putative three-dimensional structure of the nAChR confirmed that the ACh-binding site must reside at the interface between two subunits and that the ligand must penetrate into a gorge to form appropriate chemical bridges and initiate transduction (Itier and Bertrand, 2001).

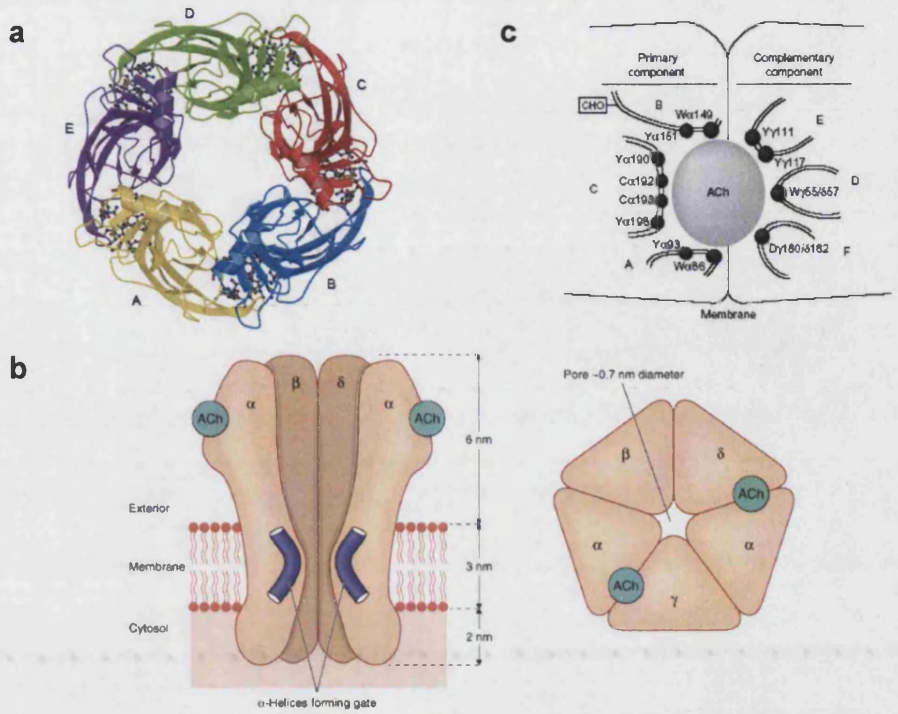


Fig. 1.12 Representation of the pentameric structure of the ACh-binding protein (a) and the ligand-binding sites of the nAChR (b, c).

(a) In this representation, each protomer has a different colour. Subunits are labelled anticlockwise, with A-B, B-C, C-D, D-E and E-A forming the principal and complimentary ligand-binding sites, respectively (ball-and-stick representation).
(b) Side- and plan-view of the muscle-type nAChR illustrates the two ACh-binding sites in the extracellular portion of the nAChR. When ACh binds, the kinked α-helices swing out of the way, thus opening the channel pore. Typical dimensions of the extracellular-, transmembrane-, and intracellular domains, and channel pore of the nAChR are depicted.
(c) Residues on the ligand-binding site, as identified by affinity labelling and site-directed mutagenesis experiments, are indicated on the primary component (on α subunits of nAChR) and complimentary component (on γ/δ subunits of muscle nAChR, or α subunits of homomeric nAChR, or β subunits of heteromeric nAChR).
(adapted from Brej et al., 2001; Changeux and Edelstein, 2001; Rang et al., 2003).

1.4.1.2 Membrane-spanning pore

The subunits in the membrane-spanning domain are each made from four α-helical segments (M1-M4). The helical segments are arranged symmetrically, forming an inner ring of helices (M2), which shape the pore, and an outer shell of helices (M1, M3 and M4), which coil around each other and shield the inner ring from the lipids (Unwin, 2005). In each of the subunits, the extracellular part of the M2 helix tilts radially inward towards its midpoint. In contrast, the other three helices tilt radially toward and tangentially around the central axis (Fig. 1.13). The water-filled cavities between the M2 helices and the outer helices are essential for the gating mechanism of the receptor as they provide the space needed for agonist-induced conformational changes in the ion channel (Miyazawa et al., 2003; Jensen et al., 2005).

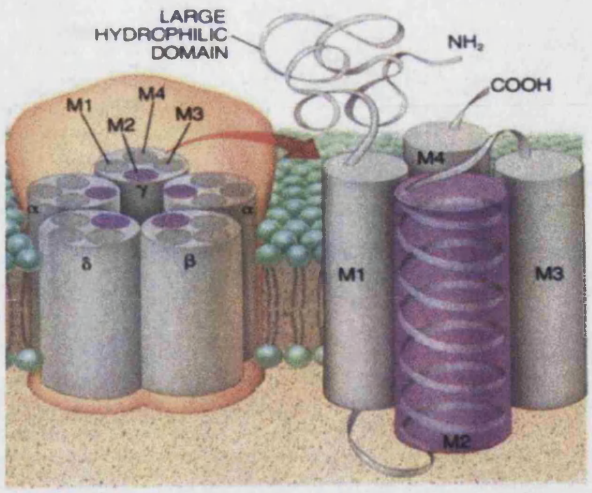


Fig. 1.13 Cross-section of the nAChR pentameric structure illustrating the transmembrane topology of the subunits.
Site-directed mutagenesis studies indicate that the M2 region of each nAChR subunit contributes to the formation of the ion pore. Each subunit has complimentary amino acids within the M2 region, which form rings within the pore (adapted from www.weizmann.ac.il).

Ion selectivity

The nAChR is a cation-selective ion channel. Residues along the entire length of the M2 domain and the N-terminal end of the M1 domain form the ion-conducting pore (Hogg et al., 2003), which discriminates between ions based on both ion charge and size. In the chick α7 receptor, the ionic selectivity of the nAChR can be converted from cationic to anionic by a minimum of three amino acid changes in the M2 domain and the M1-M2 loop (Itier and Bertrand, 2001). The channel pore comprises a tunnel of negative electrostatic potential, which favours the entry of cations (Hogg et al., 2003). When this negative charge, contributed by a ring of glutamic acid residues, is reduced by substituting glutamic acid for amino acids with uncharged side chains, the cation conductance of the nAChR is decreased (Fig. 1.14a; Hogg et al., 2003).

The gate and opening mechanism

In the resting and desensitised state of the nAChR, the ion conductance through the ion channel is blocked by a molecular barrier known as the "gate". This region is located in the middle of the M2 domain, where it is kinked in two positions (at Pro^{265} and near Leu^{251} in the α1 subunit) causing a narrowing of the ion channel (Jensen et al., 2005). The gate consists of a "leucine ring" formed by interactions between the side chains of five leucines and a "valine/isoleucine ring" one helix-turn above it (Leu^{251} and Val^{255} in the α1 subunit; Fig. 1.14a; Miyazawa et al., 2003; Unwin, 2003; Jensen et al., 2005). In the resting receptor state, the ion channel is only about 6 Å wide in this region, making permeation of hydrated cations impossible (Fig. 1.14b). In the active receptor

state, agonist binding to the N-terminal domain elicits a small (15°) axial rotation in the two α1-M2 helices, which disrupts or weakens the hydrophobic intersubunit interactions stabilising the structure around the ion channel. The relaxation of the structural constraints on the ion channel allows the five M2 helices to change orientation toward the outer helices and residues in the helices to form hydrophobic interactions with other residues in the receptor complex. As a result of this, the ion pore is widened by about 3 Å, thereby allowing cations to pass through the channel and enter the cell (Fig. 1.14b; Miyazawa et al., 2003; Unwin, 2003; Jensen et al., 2005).

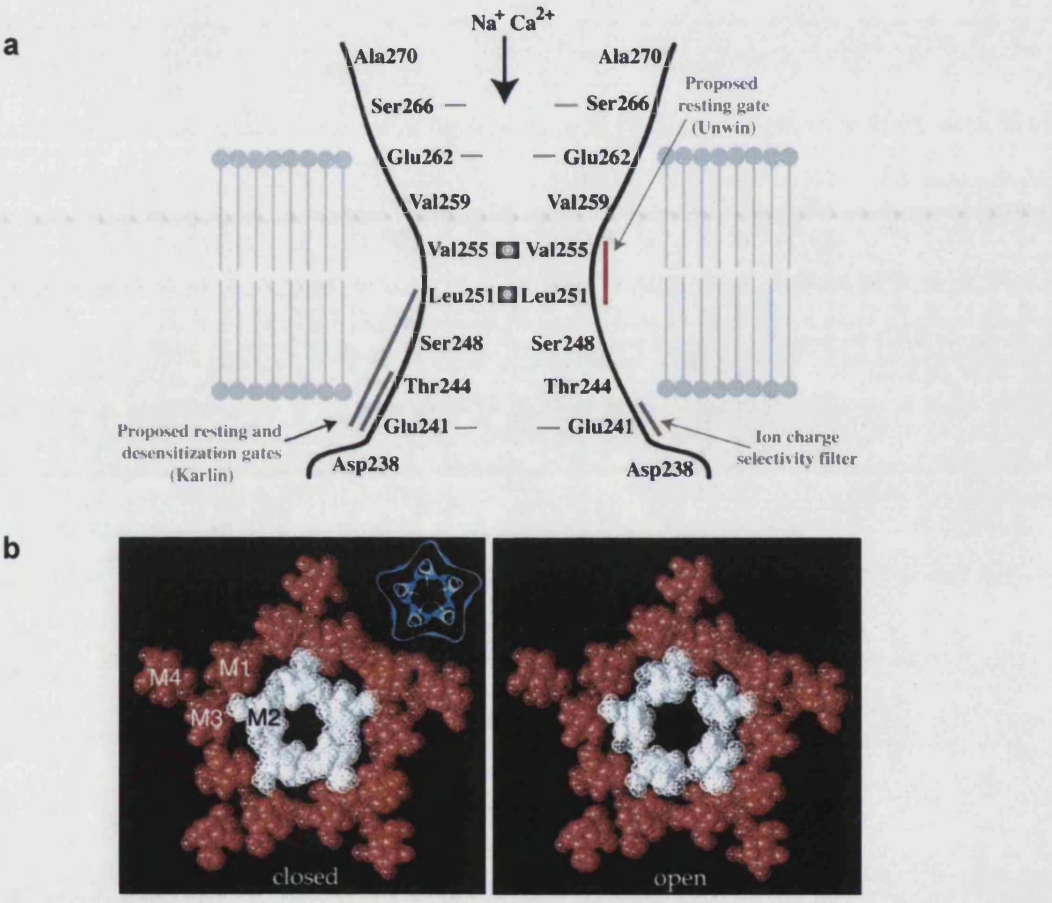


Fig. 1.14 Ion pore of the *Torpedo* nAChR.
(a) α1 nAChR residues lining the pore. The localisation of the negatively charged residues at the mouth of the ion channel, the ion charge selectivity filter, and the resting gates proposed by Unwin and Karlin are shown. The upper portion of the ion channel is believed to act as a water pore with the lower loop components acting as the selectivity filter controlling the lumenal flow of ions through the receptor.
(b) Cross sections of the nAChR in the middle of the membrane, showing the closed and open states of the nAChR gate, lined by the M2 membrane spanning segments of each subunit around a five-fold axis of pseudosymmetry. Structural changes involved in the closing (blue) and opening (white and brown) of the nAChR gate are shown in the inset.
(adapted from Unwin, 2003; Jensen et al., 2005).

1.4.2 Modes of signal transduction

The nAChR is an allosteric protein complex as it is composed of multiple subunits and contains several orthosteric (binding sites for agonists or antagonists) and allosteric sites through which its function can be modulated. Originally the nAChR was thought to open and close in a manner similar to that of an enzyme. This scheme is known as the 'sequential model'. However, evidence suggests that this transition does not occur. In 1965, the Monod-Wyman-Changeux model, termed the "concerted allosteric model", was formulated to describe the allosteric nature of nAChR signalling (Changeux et al., 1998; Changeux and Edelstein, 2001). According to this model, the nAChR fluctuates between at least three functional states in the absence of agonist: a resting state (R), an active state (A), and a desensitised state, which is divided into a fast-onset state (I) and a slow-onset (D) state (Fig. 1.15). Transitions between resting, active and desensitised states are reversible and different ligands may stabilise different receptor states. Agonists (e.g. nicotine) are characterised by having higher affinity for the active state than for the resting

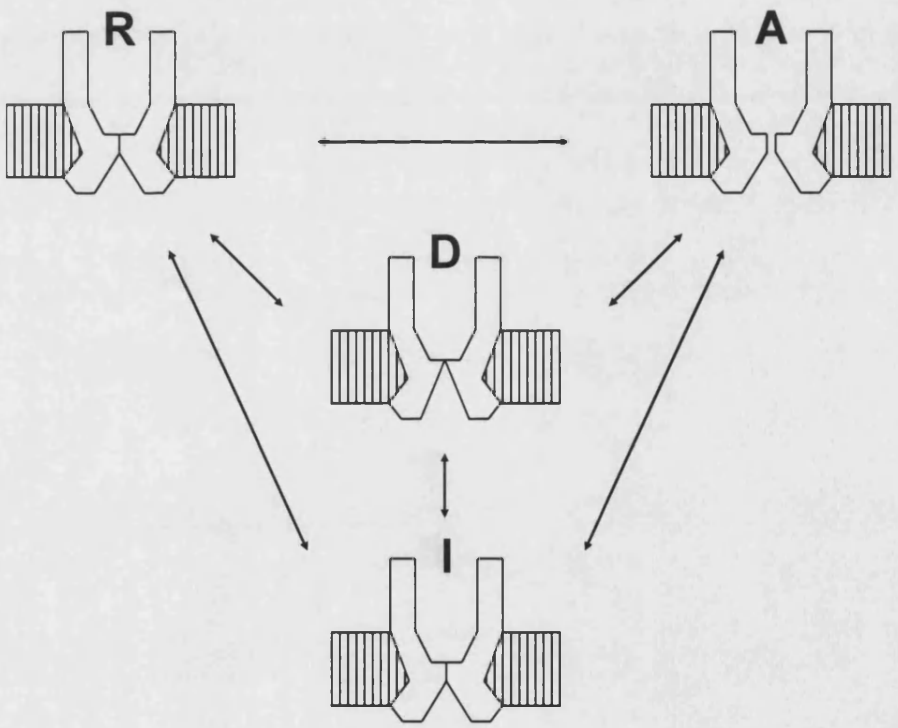


Fig. 1.15 Diagrammatic representation of the multistate allosteric nature of nAChR function.

Allosteric transitions between the resting, closed state (R), active open channel (A), and fast-onset (I) and slow-onset (D) desensitised refractory and high affinity states of the nAChR are depicted. The low-affinity resting state occurs in the absence of agonist. For the *Torpedo* nAChR, the timescales of some of the transitions are as follows: R to A = μs to ms, towards D = ms to 100 ms, towards I = up to a minute (adapted from Changeux et al., 1998).

state, and conversely, antagonists have higher affinities for resting/inactive receptor states. High-affinity binding and very high affinity binding properties characterise the fast-onset and slow-onset desensitisation states of the receptor, respectively (Changeux and Edelstein, 2001; Jensen et al., 2005).

1.4.3 Classification of nAChR

To date, 17 nAChR subunits have been identified in vertebrate species. These can be divided into three classes: (1) muscle nAChRs, (2) neuronal nAChRs that do not bind the snake venom toxin α-bungarotoxin (α-Bgt) and (3) neuronal nAChRs that do bind α-Bgt. Muscle nAChRs co-assemble into two nAChR subtypes: $(\alpha1)_2\beta1\gamma\delta$ in the foetus and $(\alpha1)_2\beta1\varepsilon\delta$ in the adult. The subunits are organised in a clockwise $\alpha1\gamma\alpha1\beta1\delta$ (or $\alpha1\varepsilon\alpha1\beta1\delta$ in adult) arrangement, and ACh binds to two orthosteric sites located at the α1-γ (or α1-ε in adult) and α1-δ interfaces of the receptor complex (Fig. 1.16; Hogg et al., 2003; Millar, 2003). Neuronal nAChRs are pentameric combinations of nine α- (α2-α10) and three β- (β2-β4) subunits (Millar, 2003; Gotti and Clementi, 2004; Jensen et al., 2005). These subunits can form a plethora of different nAChR subtypes characterised by distinct ligand pharmacology, activation and desensitisation kinetics, and cation permeability.

1.4.3.1 Neuronal nAChRs with high affinity for nicotine

A particular population of nAChRs can be pharmacologically defined by their high affinity binding to the nAChR specific agonist [^{3}H]-epibatidine which is weakly displaced, if at all, by α-Bgt (Houghtling et al., 1995). These neuronal nAChRs comprise the α2-α6 and β2-β4 subunits, which form heteropentameric nAChR complexes with a prevalent subunit stoichiometry believed to be $(\alpha)_2(\beta)_3$, arranged as αβαββ (Fig. 1.16). However, other possible stoichiometries have been demonstrated *in vitro* (Nelson et al., 2003) and may exist *in vivo* (see below for nAChR stoichiometries when they include α5 and β3 subunits). Hence, an asterisk is used in the receptor nomenclature to denote the possible presence of additional types of subunit present in the receptor complexes (Lukas et al., 1999). ACh and other orthosteric ligands, such as nicotine, bind to a site positioned at the interface between an α and a β subunit, thereby giving rise to two orthosteric sites in the nAChR.

When expressed in *Xenopus* oocytes or mammalian cell lines, α2, α3 and α4 subunits have all been shown to form functional heteromeric nAChRs in combination with β2 or β4 subunits (Chavez-Noriega et al., 1997; Stauderman et al., 1998; Lukas et al., 1999). The α6 subunit has also been shown to form functional nAChRs in combination with β2 or β4 (Gerzanich et al., 1997; Fucile et al., 1998), even though it seems to function

more effectively when combined with another α and β subunit (Fucile et al., 1998). The α5 and β3 subunits, which lack amino acid residues critical for agonist binding, do not participate in the formation of binding sites. However, together with pairs of α2-α6 and β2-β4 subunits they can form more complex nAChRs, where they become the fifth subunit not involved in orthosteric ligand binding. This is consistent with the $(\alpha4)_2\alpha5(\beta2)_2$ and $\alpha4\alpha6\alpha5/\beta3(\beta2)_2$ nAChRs inferred to reside on midbrain dopaminergic nuclei in rats (Klink et al., 2001; Wonnacott et al., 2005). The presence of either α5 or β3 subunits in nAChRs has been shown to have profound impact on its pharmacology, Ca^{2+} permeability and desensitisation kinetics (Jensen et al., 2005).

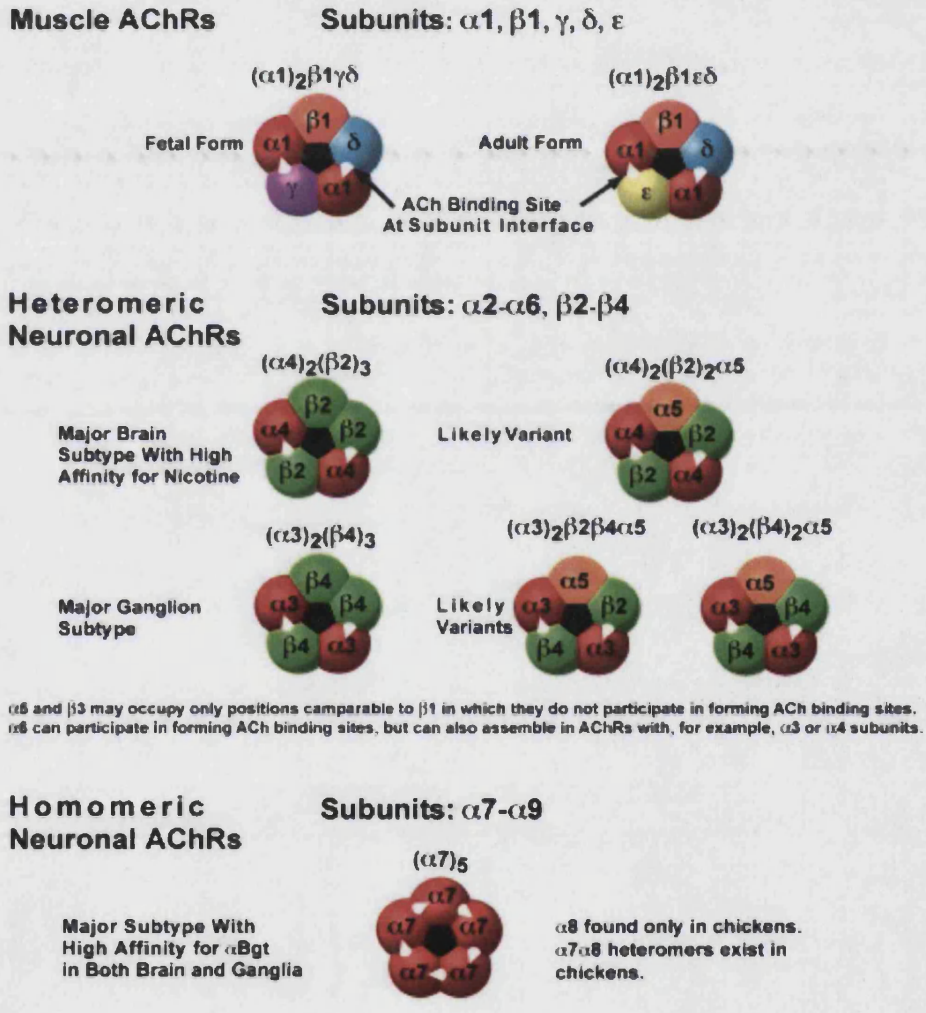


Fig. 1.16 Assembly and subunit diversity of prominent nAChR subtypes.
α-Bgt, α-bungarotoxin (adapted from www.med.upenn.edu/nscience/ neuro_lindstrom.html).

The majority of heteromeric nAChRs in the CNS (about 90%) are α4β2* receptors. The β2 subunit is expressed in almost all CNS regions, where its distribution overlaps with at least one of the α2-α4 and α6 subunits (Wada et al., 1989; Jensen et al., 2005). Although also abundantly expressed, α4 is less broadly distributed in the CNS than β2.

The highest concentrations of the α4 and β2 subunits are found in the hippocampus, thalamus and cortex (Jensen et al., 2005). In the autonomic ganglia, the most prevalent subunits are the α3 and β4, where they combine to form α3β4* nAChRs. The expression of the α2 subunit has not been studied in great detail, but it appears to be expressed in very few brain regions (Jensen et al., 2005).

1.4.3.2 Neuronal nAChRs with high affinity for α-Bgt

Unlike other neuronal nAChRs, this set of nAChRs can bind with a high affinity to α-Bgt. The α7-α9 form homopentameric receptor complexes with five orthosteric binding sites for ACh (Fig. 1.16). They are also characterised by higher relative Ca^{2+} permeabilities and faster desensitisation rates than those of the muscle-type and heteromeric neuronal nAChRs (Gotti and Clementi, 2004). Like the α4 and β2, the α7 subunit is also expressed profusely in the brain, with highest expression levels in the hippocampus and cortex (Jensen et al., 2005). However, homomeric α7 nAChRs also reside in the autonomic ganglia. The α8-containing nAChRs are only present in the chick nervous system, where they not only form homomeric receptors, but also heteromeric α7α8 nAChRs (Millar, 2003; Gotti and Clementi, 2004). Although the existence of heteromeric α7-conatining nAChRs *in vivo* is not yet available, rat α7 subunits have been shown to co-assemble with β2 subunits, expressed in heterologous systems (Khiroug et al., 2002).

The α9 subunit is expressed in bone marrow, nasal epithelium and embryonic blood cells, and co-expressed with α10 in cochlear outer hair cells (Jensen et al., 2005). The α10 subunit is similar to the α9 subunit by amino acid sequence, but is not able to form homomeric nAChRs. The α10 subunit can only form a functional nAChR together with the α9 subunit. However, the exact stoichiometry and arrangement of the α9α10 nAChR in unknown. Both α9 and α9α10 nAChRs are characterised by mixed nicotinic/muscarinic pharmacological profile (Gotti and Clementi, 2004; Jensen et al., 2005).

1.4.4 Cellular localisation of nAChR

The great diversity of neuronal nAChRs is consistent with their varied roles. In the muscle and autonomic ganglia, nAChRs are located pre- and post-synaptically. In their post-synaptic locations, nAChRs mediate fast synaptic transmission. The most well studied example of this is at the neuromuscular junction, which is the synapse between a motor neurone and skeletal muscle. Upon arrival of an action potential, the presynaptic

motor neurone releases ACh, which diffuses across the synaptic cleft and activates nAChRs on the postsynaptic muscle. Fast nicotinic transmission has been reported in the brain only rarely. Part of the reason is due to the difficulty in demonstrating such a function, as cholinergic neurones in the brain are not tightly packed and often make broad, diffuse projections, making it complex to stimulate and record from a precise location of neuronal innervation. In addition to this, the high Ca^{2+} permeability of neuronal nAChRs has kindled the notion that nAChRs may serve to modulate (rather than mediate) the release of ACh and other neurotransmitters in the CNS (Role and Berg, 1996; Wonnacott, 1997). Facilitation of ACh release by nAChR activation is known as autoregulation, whereas for other neurotransmitters, it is termed heterosynaptic modulation (Wonnacott, 1997; MacDermott et al., 1999; Hogg et al., 2003).

On the basis of electrophysiological and transmitter release studies, it has become a common perception that neuronal nAChRs are predominantly presynaptic (Fig. 1.17; Role and Berg, 1996; Wonnacott, 1997) and reside on the membranes of nerve terminals. Various functional and ultrastructural studies have suggested that neuronal nAChRs may also exist on preterminal membranes (Fig. 1.17; Wonnacott, 1997). These nAChRs are located before the presynaptic terminal bouton and can indirectly modulate neurotransmitter release by activating voltage-gated channels that consequently induces neurotransmitter release from the presynaptic terminal. The evidence is strongest in GABAergic synapses where preterminal nAChR activation depolarises the membrane locally, thereby activating voltage-gated channels that directly mediate the presynaptic Ca^{2+} influx underlying enhanced GABA release (Dani and Lester, 2001). This effect of preterminal nAChRs is inhibited by tetrodotoxin (TTX), which blocks sodium channels, thereby preventing the regenerative voltage-dependent activation of Ca^{2+} channels in the terminal, which subsequently inhibits the release of neurotransmitter from the presynaptic terminal. In contrast, presynaptic nAChRs modulate neurotransmitter release directly, or influence the probability of an action potential resulting in exocytosis (Wonnacott, 1997). Hence, these nAChRs are Ca^{2+}-dependent but TTX-insensitive. However, studies of striatal synaptosomes (defined as presynaptic elements – see section 2.1.1.1; Marks et al., 1995; Marshall et al., 1996) have challenged this simple distinction, by reporting a partial block by TTX of nicotine-evoked rubidium ($^{86}Rb^{+}$) or [^{3}H]DA release. TTX-sensitive and insensitive mechanisms might reflect the relative proximity of nAChRs to the synapse and exocytotic machinery. Thus, TTX-sensitivity alone does not necessarily distinguish preterminal and presynaptic nAChRs. In addition to provoking Ca^{2+}-dependent exocytosis, presynaptic nAChRs also modulate neurotransmitter release through Ca^{2+}-mediated cellular mechanisms (Dajas-Bailador and Wonnacott, 2004).

Following exogenous application of nicotinic agonists, presynaptic nAChRs can directly modulate the Ca^{2+}-dependent release of many neurotransmitters such as ACh, DA, NA, 5-HT, glutamate and GABA (Role and Berg, 1996; Wonnacott, 1997). In many

cases, the α-Bgt-sensitive nAChRs are responsible for regulating glutamatergic neurotransmission (Mansvelder et al., 2002), but in other cases different nAChR subtypes are involved (Dani and Lester, 2001). The view that dopaminergic transmission is central to reward mechanisms makes the associated nAChRs the conduit for the delivery of signals that instigate and maintain nicotine addiction (Wonnacott et al., 2005).

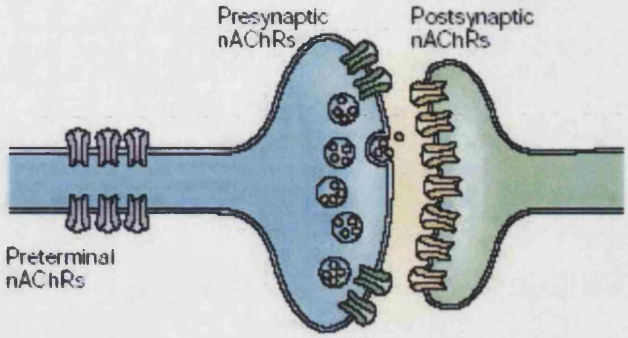


Fig. 1.17 Representation of nAChRs at synaptic locations.
Presynaptic nAChRs are located on presynaptic boutons, where they can influence the release of neurotransmitter that is present in synaptic vesicles by depolarising the nerve terminal and activating Ca^{2+}-dependent exocytosis. Preterminal nAChRs are located on the axon before the synaptic terminal, where they can mediate a depolarisation locally to activate voltage-operated Ca^{2+} channels that consequently induces neurotransmitter release from the presynaptic terminal (adapted from Laviolette and van der Kooy, 2004).

1.4.5 Distribution of nAChRs in midbrain DA systems relevant to nicotine addiction

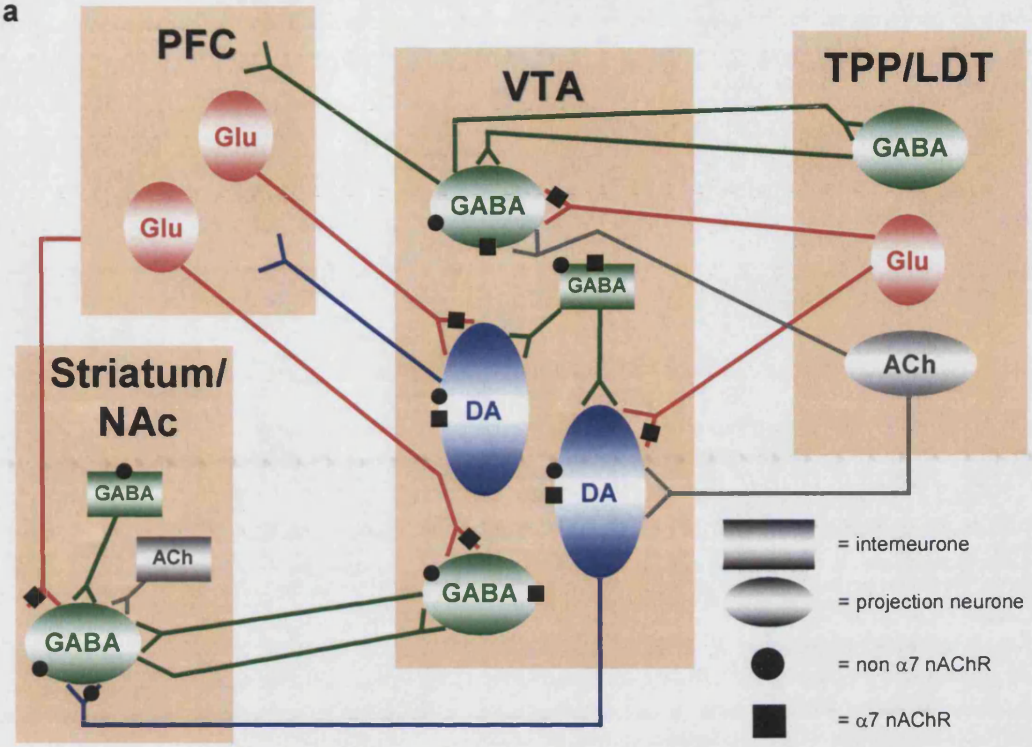
Although the function of numerous neurotransmitter systems expressing nAChRs can be altered by nicotine, the initial reinforcing properties of this alkaloid are thought to be mediated primarily through the mesocorticolimbic DA system. In brief, these neuronal pathways consist of DA neurones from the VTA that project to the NAc (ventral striatum) and other forebrain sites, such as the prefrontal cortex (PFC; see section 1.2.2). Brain areas in this system express a variety of nAChR subtypes, which are the major substrate of nicotine reward and reinforcement (Pidoplichko et al., 2004; Balfour, 2004; Wonnacott et al., 2005).

1.4.5.1 Ventral Tegmental Area

The VTA consists of three cell types shown to express nAChRs: DA neurones, GABA neurones and glutamatergic afferents (Fig. 1.18a; Klink et al., 2001; Mansvelder and McGehee, 2000; Champtiaux et al., 2002; Champtiaux et al., 2003; Wonnacott et al., 2005). Most nAChR subunits (α3-α7 and β2-β4) are expressed in the VTA resulting in

three pharmacologically identifiable nAChRs: one that is likely to be a homomeric α7 nAChR and two that do not contain the α7 subunit (Klink et al., 2001; Champtiaux et al., 2002; Champtiaux et al., 2003).

a



b

Brain region	nAChR subtype	Neuronal type
VTA	$(\alpha4)_2(\beta2)_3$, $(\alpha7)_5$	GABAergic
	$\alpha4\alpha6\alpha5/\beta3(\beta2)_2$, $(\alpha4)_2\alpha5(\beta2)_2$, $(\alpha7)_5$	Dopaminergic
Striatum	$(\alpha4)_2(\beta2)_3$, $(\alpha4)_2\alpha5(\beta2)_2$,	Dopaminergic
	$(\alpha6)_2(\beta2)_2\beta3$, $\alpha4\alpha6(\beta2)_2\beta3$	

Fig. 1.18 The mesocorticolimbic DA system, associated circuitry and nAChR subtypes.

(a) A simplified schematic of afferent and efferent connections between the ventral tegmental area (VTA), striatum/nucleus accumbens (NAc), prefrontal cortex (PFC) and tegmental pedunculopontine nucleus/laterodorsal tegmental nucleus (TPP/LDT) is depicted. The major pathways using dopamine (DA), γ-aminobutyric acid (GABA), acetylcholine (ACh) and glutamate (Glu), including the locations of α7- and β2-containing nAChRs, are illustrated.

(b) Summary of the somatodendritic nAChR subtypes proposed to reside on dopaminergic and GABAergic neurones in the VTA and presynaptic nAChRs on dopaminergic boutons in the terminal field.

(adapted from Wonnacott et al., 2005).

DA neurones

A majority of DA neurones express nAChRs that can be blocked by mecamylamine (a general nicotinic antagonist), whereas less than half of the DA

neurones express α7 nAChRs (Klink et al., 2001; Wooltorton et al., 2003). The α6 and β3 subunits are also expressed by DA cells, and recent studies using transgenic mice lacking either of these subunits show that they predominate in presynaptic, relative to somatodendritic, nAChRs (Champtiaux et al., 2003; Cui et al., 2003). The subunit compositions $(\alpha4)_2\alpha5(\beta2)_2$ and $\alpha4\alpha6\alpha5(\beta2)_2$ nAChRs are proposed to reside on DA cell bodies (Fig. 1.18b; Klink et al., 2001), and these nAChRs occur in the ratio 80:20, respectively (Champtiaux et al., 2003). The β3 subunit may replace the α5 subunit in a proportion of somatodendritic nAChRs (Cui et al., 2003). Functional α7 nAChRs have also been found to exist on DA neurones (Fig. 1.18b; Klink et al., 2001; Wooltorton et al., 2003).

GABA neurones

A population of GABA interneurones provides local inhibitory input to the mesocorticolimbic DA neurones (Fagen et al., 2003; Wonnacott et al., 2005), and there is anatomical evidence for descending GABAergic projections to the brainstem mesopontine region (Semba and Fibiger, 1992): the tegmental pedunculopontine nucleus (TPP), which has been implicated in various aspects of nicotine-induced DA-independent drug seeking and reward (Corrigall et al., 2002; Laviolette et al., 2002; Laviolette and van der Kooy D., 2004), and the adjacent laterodorsal tegmental nucleus (LDT). In addition to receiving GABAergic inputs from the TPP and NAc, the VTA sends GABAergic projections to the NAc and PFC (Wonnacott et al., 2005). GABA neurones in the VTA express a similar variety of nAChR subunits, but in contrast to DA neurones, α5, α6, β3 and β4 were found in less than 25% of the GABA neurones (Klink et al., 2001). Thus, the majority of VTA GABA neurones have been suggested to express predominantly $(\alpha4)_2(\beta2)_3$ nAChRs (Fig. 1.18b; Champtiaux et al., 2003), which are blocked by mecamylamine and dihdro-β-erythroidine (DHβE), a β2* nAChR-selective antagonist (Mansvelder et al., 2002). The remaining GABA neurones are considered to contain α7 nAChRs (Fig. 1.18b; Klink et al., 2001).

Glutamatergic afferents

The VTA receives glutamatergic synaptic input primarily from the PFC, which has been suggested to provide the major excitatory control of VTA neurone activity and ultimately DA release in the NAc (Mansvelder and McGehee, 2002). However, the glutamatergic projections from the PFC do not synapse onto DA neurones that project to the NAc. Rather, they make contact with GABAergic neurones that project to the NAc and DA neurones that project back to the PFC (Carr and Sesack, 2000). Ascending inputs from the TPP and LDT to the VTA comprise cholinergic and glutamatergic fibres that synapse onto DA and GABA neuronal populations of the VTA (Garzon et al., 1999). The

cholinergic neurones of the mesopontine nuclei can modulate the activity of the mesolimbic system, since electrical stimulation of the TPP elicits striatal DA efflux (Forster and Blaha, 2003), whereas LDT stimulation evokes a similar DA overflow in the NAc through the activation of cholinergic and glutamatergic receptors in the VTA (Forster and Blaha, 2000). This is consistent with glutamatergic afferents from the LDT that preferentially innervate NAc-projecting VTA DA neurones (Kauer, 2004). Furthermore, the non-α6 α4β2* nAChRs on VTA DA neurones are thought to mediate most of the slow ACh-elicited currents in the somatodendritic compartment (Zoli et al., 2002; Champtiaux et al., 2003). Histochemical labelling has recently confirmed the presence of α7 nAChRs on glutamatergic terminals in the VTA (Fig. 1.18b; Jones and Wonnacott, 2004), where they serve to enhance long-term potentiation (LTP) onto DA neurones (Mansvelder et al., 2002).

1.4.5.2 Striatum vs. Nucleus accumbens

The word striatum is derived from the Latin *stria*, meaning a furrow, and is due to the large number of myelinated axons that run the length of the region, giving it a conspicuous, lined appearance. The striatum belongs to a group of anatomically distinct structures that are collectively known as the basal ganglia. Based on the connectivity and function, the striatum is often divided into dorsal and ventral portions. The dorsal striatum comprises the caudate-putamen, and is mainly sensorimotor related. It receives DA innervation primarily from the SNc and to a lesser degree from the VTA (see section 1.2.1). The medial and lateral areas of the dorsal striatum receive glutamatergic inputs preferentially from associative- and sensorimotor-related cortical areas (Gerdeman et al., 2003). The principal neurones of the striatum are the medium-sized spiny projection neurones, which make up about 90-95% of the total striatal population. They possess large dendritic trees

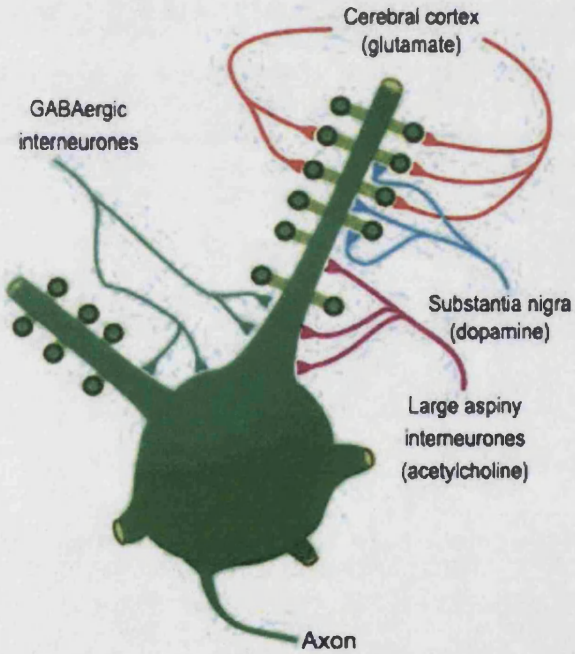


Fig. 1.19 Diagrammatic representation of the termination of afferents on a medium-sized spiny neurone in the striatum.
Dopamine (blue), glutamate (red), acetylcholine (pink) and GABAergic (light green) inputs are illustrated (adapted from Hyman and Malenka, 2001).

and extensive local axon collaterals (Fig. 1.19). These neurones are inhibitory, using GABA as their primary neurotransmitter. The remaining minority of neurones are the GABAergic and cholinergic interneurones (Fig. 1.18a; Zhou et al., 2003). Part of the medium-sized spiny GABAergic projection neurones in the dorsal striatum project to the internal segment of the GP and the SN pars reticulata, forming the so-called "direct pathway". Other striatal medium-sized spiny neurones project to the external segment of the GP and are part of the "indirect pathway" (Zhou et al., 2003).

The ventral striatum is composed partly of the NAc and portions of the olfactory tubercule, and is mainly limbic related. It receives DA innervation primarily from the VTA and to a lesser degree from the SNc. The NAc sends inhibitory projections to the VTA and surrounding DA areas, and receives extensive glutamatergic innervation from the PFC, hippocampus and amygdala (Fig. 1.18a; Zhou et al., 2002). DA neurones in the NAc form synapses at the neck of the medium-sized spiny neurones, whilst glutamatergic projections tend to be associated with the head of the spines (Fig. 1.19; Wightman and Robinson, 2002).

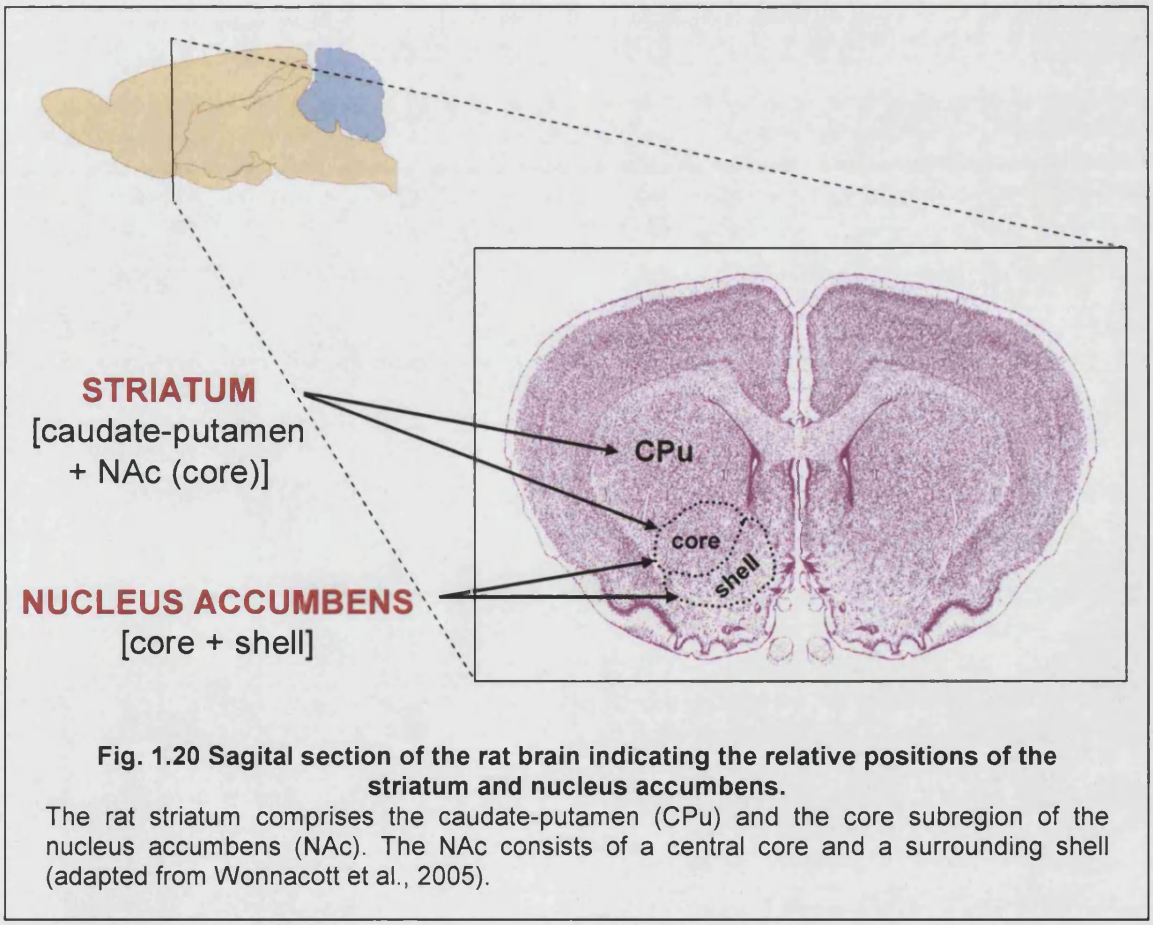


Fig. 1.20 Sagital section of the rat brain indicating the relative positions of the striatum and nucleus accumbens.
The rat striatum comprises the caudate-putamen (CPu) and the core subregion of the nucleus accumbens (NAc). The NAc consists of a central core and a surrounding shell (adapted from Wonnacott et al., 2005).

The NAc consists of two major subdivisions: a central core (NAc_{core}), which is part of the ventral striatum (Fig. 1.20), and a surrounding shell (NAc_{shell}), which forms part of the limbic system, anatomically being an extension of the amygdala (Voorn et al., 2004). The two accumbal sectors differ in their efferent projections. The NAc_{core} circuit involves

projections to the dorsolateral ventral pallidum (VP), which in turn projects to the subthalamic nucleus (STN) and SNc (Deutch and Cameron, 1992), the main source of DA innervation of the striatum. The NAc_{shell} circuit is characterised by projections to the ventromedial VP, which does not innervate the STN but projects to the VTA (Deutch and Cameron, 1992), the primary site of midbrain DA neurones that innervate mesocorticolimbic areas (see section 1.2.2). Since the NAc_{core} resembles more closely the dorsal striatum and sends major projections to areas of the brain concerned with the regulation of motor function, it seems to play a pivotal role in responding that is elicited, or reinforced, by conditioned stimuli (Ito et al., 2004; Wonnacott et al., 2005; see section 1.1). The NAc_{shell}, being part of the mesolimbic sector, is involved in the acquisition of responding (such as lever pressing for drug) for motivationally significant stimuli (Di Chiara, 2002; Wonnacott et al., 2005).

Various experimental strategies applied to striatal preparations (the NAc being too small for such biochemical analyses) have resulted in the consensus concerning presynaptic nAChR subtypes in the terminal fields. Histochemical and ion flux measurements with synaptosomes have indicated that nAChRs are expressed on all DA axon terminals in the striatum (Jones et al., 2001). Co-immunoprecipitation experiments in conjunction with ligand binding (Champtiaux et al., 2002; Champtiaux et al., 2003) and following 6-hydroxydopamine lesions of the dopaminergic afferents (Zoli et al., 2002) provided evidence for subunit composition of nAChRs in the striatum. Further support came from pharmacological analysis of nAChR-evoked DA release (Salminen et al., 2004), with comparisons of transgenic mice lacking a particular nAChR subunit. Together, these studies have defined four populations of nAChRs in the rodent striatum: $\alpha4\beta2$ nAChRs, which are expressed on both dopaminergic and non-dopaminergic neurones; $\alpha4\alpha5\beta2$, $\alpha6\beta2\beta3$ and $\alpha4\alpha6\beta2\beta3$, which are only expressed on dopaminergic terminals (Fig. 1.18b). Electron microscopy coupled with immunocytochemistry revealed that all striatal DA axon boutons express the $\beta2$ nAChR subunit (Jones et al., 2001). The significance of the heterogeneity, and whether nAChR subtypes and segregated or co-localised, is not yet understood. Presynaptic $\alpha7$ nAChRs have not been detected on dopaminergic terminals, but they have been inferred to reside on glutamatergic afferents projecting to the striatum (Kaiser and Wonnacott, 2000).

1.4.6 Nicotinic modulation of DA release

As previously stated (see section 1.2.2), it is believed that the reinforcing properties of nicotine are due to its capacity to modulate the mesolimbic DA system (Corrigall et al., 1992). Like many drugs of abuse, nicotine exerts it addictive properties by increasing DA release in the NAc, predominantly in the NAc_{shell} (Imperato et al., 1986;

Balfour et al., 1998). This is seen after systemic injections of nicotine, or local application of nicotine directly into the VTA or NAc, which stimulates DA overflow in the NAc (Mifsud et al., 1989; Marshall et al., 1997; Ferrari et al., 2002). However, local infusion of non-specific nicotinic antagonists into the VTA prevents nicotine-induced DA release in the NAc (Nisell et al., 1994a; Nisell et al., 1994b) and disrupts systemic nicotine self-administration in rats (Corrigall et al., 1994), whereas perfusion of the NAc with these antagonists has no effect on either phenomenon (Corrigall et al., 1994; Nisell et al., 1994a). Therefore, the gross DA responses in the NAc to systemic nicotine have been attributed, at least in part, to the direct activation of nAChRs located on DA neurones of the VTA.

1.4.6.1 Burst firing of DA neurones

Under ambient behavioural conditions, DA neurones fire tonically or irregularly at frequencies around 4 Hz, but when activated by salient or arousing sensory input, DA neurones begin to burst fire (Cooper, 2002). Hence, phasic burst firing (>10 Hz) rather than single-spike firing (3-5 Hz) represents a more efficient means of increasing NAc or striatal DA concentrations (Gonon, 1988). Systemic injections of nicotine increase the proportion of midbrain DA neurones that exhibit burst firing (Nissbrandt et al., 1994; Nisell et al., 1996). The supralinear increase in extracellular DA concentration is thought to arise from a combination of factors such as rapid saturation of DAT, slow enzymatic breakdown of DA, and slow DA autoreceptor activation, all of which usually work together to reduce extrasynaptic DA (Cooper, 2002).

1.4.6.2 β2* nAChRs mediate the reinforcing properties of nicotine

Intravenous nicotine self-administration in laboratory animals is one of many reliable behavioural measures of the reinforcing properties of nicotine. Transgenic mice lacking the β2 nAChR subunit do not self-administer nicotine (Picciotto et al., 1998; Epping-Jordan et al., 1999), whereas responding for cocaine is unaffected (Epping-Jordan et al., 1999). Additionally, nicotine self-administration is blocked by DHβE, but not by the α7-selective antagonist methylcaconitine (MLA; Grottick et al., 2000). Electrophysiological experiments demonstrate that nicotine fails to increase the firing rate of somatic DA neurones in mice lacking the β2 nAChR subunit (Picciotto et al., 1998). Nicotine-evoked increase in extracellular DA is abolished in striatal synaptosomes from β2 knock-out animals (Picciotto et al., 1998; Grady et al., 2001). These observations demonstrate that nicotine reinforcement is dependent on the activation of β2* nAChRs.

Various studies have been conducted to determine the nature of nAChR subunits likely to be associated with the β2 subunit. Based on pharmacological data, the α4 and α3 subunits have been proposed to partner with the β2 subunit on DA terminals (Sharples et al., 2000). Systemic nicotine-evoked DA release, *in vivo*, is abolished in α4 knock-out mice (Marubio et al., 2003). Also, α4* nAChRs have been shown to be sufficient for nicotine-induced reward, tolerance and sensitisation (Tapper et al., 2004). Conversely, the role of the α3 nAChR subunit in nicotine reinforcement has been challenged. Initially, the contribution of the α3 subunit had been proposed in view of its selectivity of α-conotoxin MII (αCtxMII) for recombinant α3β2* nAChRs expressed in *Xenopus* oocytes and the discovery that this toxin partially inhibits nicotine-evoked DA release in striatal synaptosomes (Kulak et al., 1997; Kaiser et al., 1998). However, this selectivity has been questioned by the observation that high affinity αCtxMII binding sites are preserved in α3 knock-out animals but completely disappear in α6 knock-out mice (Champtiaux et al., 2002; Whiteaker et al., 2002), suggesting that the α6 rather than the α3 nAChR subunit in DA neurones is likely to associate with the β2 subunit. Altogether, these results reveal that α4 and α6 subunits are most likely to associate with the β2 subunit to form functional heteromeric nAChRs on DA neurones (Champtiaux and Changeux, 2004; see section 1.4.5 for nAChR subtypes on midbrain DA neurones).

1.4.6.3 Acute effects of nicotine

Nicotine effects in the VTA

Microdialysis studies in rats have shown that a single injection of systemic nicotine produces a sustained elevation of extracellular DA in the NAc for 60-120 min (Imperato et al., 1986; Di Chiara, 2000; Ferrari et al., 2002) and subsequent injections of the drug in the 90 min period following a nicotine injection have little additional effect on DA overflow (Balfour et al., 2000). During cigarette smoking, blood nicotine levels reach 300-500 nM within minutes after the initial exposure to nicotine, and concentrations close to 250 nM are sustained for 10 min or more thereafter (Henningfield et al., 1993). Therefore, such a time course of nicotine would activate high-affinity nAChRs on DA neurones in the VTA, which however, desensitise in seconds to minutes when exposed to nicotine (Fig. 1.15; Pidoplichko et al., 1997; Wooltorton et al., 2003), and thus cannot account for the long lasting increase in DA release observed *in vivo* upon a single nicotine exposure. Mansvelder and colleagues (Mansvelder and McGehee, 2000; Mansvelder et al., 2002) argue that the persistent effects of nicotine on DA release in the NAc may be explained by nicotine-induced LTP of the excitatory glutamatergic input and nicotine-induced depression of GABAergic transmission within the VTA.

Previous work has indicated that α7 nAChRs residing on glutamatergic terminals (Jones and Wonnacott, 2004) may regulate the release of glutamate in the VTA, as this effect is blocked by MLA (Schilstrom et al., 2000b). Furthermore, antagonism of NMDA receptors on DA neurones and α7 nAChRs in the VTA diminishes nicotine-induced DA overflow in the NAc (Shoaib et al., 1994; Schilstrom et al., 1998a; Schilstrom et al., 1998b; Schilstrom et al., 2000a). Hence, when nicotine arrives in the VTA, it stimulates nAChRs on both presynaptic glutamatergic terminals and on DA neurones, thereby favouring conditions of paired electrical stimulation of pre- and postsynaptic partners to potentiate excitatory transmission (Mansvelder et al., 2003). Since nicotine excites and depolarises VTA DA neurones, the magnesium block of the NMDA receptors is removed. Simultaneously, nicotine enhances the release of glutamate via presynaptic α7 nAChRs, which assists further postsynaptic NMDA receptor activation, and so is more likely to produce LTP of the glutamatergic afferents onto DA neurones (Dani et al., 2001; Mansvelder and McGehee, 2002). Low concentrations of nicotine associated with tobacco use do not induce significant desensitisation of α7 nAChRs (Mansvelder et al., 2002; Wooltorton et al., 2003), as compared to the non-α7 nAChRs (Mansvelder and McGehee, 2002). This allows continuous enhancement of glutamate release onto DA neurones in the VTA whilst nicotine is present.

In addition to excitatory inputs, VTA DA neurones are under inhibitory control, predominantly by GABAergic interneurones and also by projecting GABA-fibres from other brain areas (see section 1.4.5.1). When nicotine reaches the VTA, nAChRs expressed by GABA neurones are activated and cause an increase in the firing rate of these neurones (Mansvelder et al., 2002), which produces a transient increase in the inhibitory input to the DA neurones in the VTA. This effect is likely to offset some of the excitatory effects of nicotine during the initial stages of nicotine exposure. However, similar to the α4β2* nAChRs on the DA neurones, GABA neurones in the VTA also express α4β2 nAChRs (Fig. 1.18b). These nAChRs undergo rapid desensitisation within minutes after the initial exposure to low concentrations of nicotine. Consequently, the inhibitory input to VTA DA neurones is diminished, making them more active (Mansvelder et al., 2003). Cholinergic inputs to the VTA predominantly target non-DA neurones (Garzon et al., 1999), and stimulation of these cholinergic inputs increase GABAergic transmission. This is evident when breakdown of ACh in the VTA is inhibited with an inhibitor of acetylcholinesterase (AChE; Mansvelder et al., 2002). Therefore, desensitisation of the nAChRs of the VTA GABA neurones not only prevents further activation by nicotine, it also precludes their contribution to endogenous cholinergic transmission (Mansvelder et al., 2002; Mansvelder et al., 2003). Through the differential expression of nAChR subtypes and a combination of nicotine-induced enhanced glutamatergic and depressed GABAergic transmission onto VTA DA neurones, there is a net shift towards stimulation of the mesolimbic DA pathway

and subsequent maintenance of elevated extracellular DA in the NAc (Laviolette and van der Kooy D., 2004). This hypothesis (Mansvelder et al., 2002) is compatible with the proposed role for burst firing in the mechanism underlying increases in DA overflow in the NAc evoked by nicotine.

Nicotine effects in the striatum

The striatal DA terminals and cholinergic interneurones form an intertwined meshwork that allows DA and ACh to spill over from their synapses into the extracellular space, thus contributing to volume transmission. The cholinergic interneurones provide an ongoing ACh signal by firing action potentials tonically at about 2-5 Hz (Zhou et al., 2002; Mansvelder et al., 2003). The ACh in the extracellular space influences neighbouring DA terminals by activating presynaptic nAChRs, thereby enhancing the probability of DA release when an action potential arrives along the axon of the DA terminal (Role and Berg, 1996; Wonnacott, 1997; Zhou et al., 2001). The dense AChE rapidly degrades ACh and terminates the ACh signal so that the nAChRs are not strongly desensitised. Consequently, DA terminals are not overdriven by prolonged nAChR activation, and nAChR desensitisation is also kept low. Although the principal feature of nicotine addiction is to increase DA levels in the NAc via an initial effect in the VTA, downstream cholinergic modulation of nicotine-evoked DA release in the NAc helps to synchronise and co-ordinate the influence of DA over striatal output neurones.

1.4.6.4 Chronic effects of nicotine

Repeated daily exposure to systemic nicotine results in increased DA overflow in the NAc in response to a subsequent systemic nicotine challenge compared with drug naïve rats (Benwell and Balfour, 1992). This sensitised response is characteristic of other drugs of dependence and is implicated in the neurobiology underlying the transition to dependence (Robinson and Berridge, 1993). The sensitised responses to acute systemic challenges with nicotine are antagonised by local infusions of mecamylamine into the VTA (Nisell et al., 1994b; Balfour et al., 1998). The locomotor stimulant effect of nicotine, which is blocked by lesions of the mesolimbic DA system (Clarke et al., 1988), has been shown to be more pronounced when nicotine is infused locally into the VTA (Reavill and Stolerman, 1990). Furthermore, locomotor sensitisation to nicotine has been demonstrated after chronic intermittent treatment with nicotine administered systemically as well as when nicotine was microinjected into the VTA (Reavill and Stolerman, 1990; Panagis et al., 1996; Domino, 2001). Sensitisation to systemic nicotine is prevented following co-administration of an NMDA receptor antagonist with nicotine (Shoaib et al., 1994; Domino, 2001), implicating a role of NMDA receptors in nicotine-induced sensitisation. Together, these results point to a key role of nAChRs located in the VTA

region to induce sensitisation to nicotinic effects following chronic administration. However, chronic nicotine treatment (systemic injections of 0.4 mg/kg nicotine for 7 days or 4 mg/kg/day of nicotine via osmotic mini-pumps for 7 days) further enhances mecamylamine-sensitive DA release in response to local infusion of nicotine into the NAc and striatum (Marshall et al., 1997). This suggests that nAChRs expressed at synaptic terminals of midbrain DA neurones are also likely to contribute to the development of mesolimbic DA system sensitisation to nicotinic effects.

Sensitised DA overflow in response to nicotine is regionally dependent, being observed in the NAc_{core} but not in the NAc_{shell}, whilst acute nicotine injections in drug-naïve animals preferentially stimulates DA overflow in the NAc_{shell} (Benwell and Balfour, 1992; Cadoni and Di Chiara, 2000; Iyaniwura et al., 2001). Furthermore, sensitisation of nicotine-induced DA efflux in the NAc_{core} is only observed in rats pretreated with low or moderate doses of nicotine that may be rewarding and not in animals pretreated with high, aversive doses of the drug (Iyaniwura et al., 2001). There is growing consensus that the two principal subdivisions of the NAc play complementary roles in the neurobiology underlying drug dependence (Balfour, 2004; Voorn et al., 2004).

Di Chiara (2002) has proposed an associative learning hypothesis that posits that the substantial and sustained increases in DA overflow in the NAc, evoked by psychostimulant drugs of dependence such as nicotine, result in the stimuli associated with delivery of the drug acquiring powerful motivational properties at the expense of non-drug cues (Di Chiara, 2002). These incentives are known as conditioned stimuli (see section 1.1). The hypothesis proposes that substantial and sustained increases in extracellular DA, evoked by psychostimulant drugs, greatly enhance the probability that animals will learn and repeat behaviours that result in delivery of the drug and the predictive nature of stimuli that are associated with these behaviours. Non-contingent presentation of a conditioned stimulus evokes a regionally selective increase in DA overflow within the NAc_{core} and stimulates drug-seeking behaviour (Ito et al., 2000). Regionally selective excitotoxic lesions of neurones in the NAc_{core} (but not the NAc_{shell}) significantly impair the influence of conditioned stimuli on the acquisition and maintenance of drug-seeking behaviour (Ito et al., 2004). Therefore, the primary consequence of the increases in DA overflow evoked by such drugs in the NAc_{core} may be the compulsive or Pavlovian drug-seeking behaviour, in response to cues or stimuli associated with delivery of the drug, that characterise the transition to dependence (Ito et al., 2000; Balfour, 2004).

Long-term nicotine exposure causes an upregulation of nAChR (Wonnacott, 1990) with a high affinity for nicotine, namely the $\alpha4\beta2^*$ nAChRs (Buisson and Bertrand, 2002), as they are important for nicotine self-administration (Picciotto et al., 1998). Previously, it had been suggested that upregulation of $\alpha4\beta2$ nAChRs was caused by numerous processes that increased the number of surface nAChRs (Buisson and Bertrand, 2002). However, recent studies by Vallejo and colleagues (2005) propose that nicotine exposure

may, in fact, slowly stabilise α4β2 nAChRs in a high-affinity state that would increase both nicotine binding, without altering the number of receptors, and the response of the nAChR to nicotine (Vallejo et al., 2005). Hence, it is possible to speculate about a common pattern of cigarette smoking. At nicotine levels achieved by smokers, α4β2 nAChRs in the brain initially cycle through one set of resting, activated, and desensitised states (Fig. 1.15). With continued smoking, the same α4β2 nAChRs would slowly shift into the upregulated condition with different resting, activated and desensitised states. The increased response and sensitivity to nicotine, which are characteristic of the upregulated nAChR, and the slow entry and exit from the upregulated state, should provide a long-lasting memory of the presence of nicotine in neurones containing the α4β2 nAChRs (Vallejo et al., 2005). Therefore, upregulation of α4β2* nAChRs on presynaptic DA-releasing terminals of the mesolimbic pathway may lead to an enhanced presynaptic depolarisation and an increased release of DA. This sensitised effect (Balfour et al., 1998) together with the process of nAChR upregulation, which is likely to affect various nAChR subtypes differently, could influence synaptic plasticity (Mansvelder and McGehee, 2000; Dani et al., 2001) that contributes to the long-term changes associated with nicotine addiction.

1.5 Smoking cessation

Based on the evidence that nicotine has the properties of a psychostimulant drug of dependence, a majority of pharamcotherapies for tobacco dependence have been targeted on the hypothesis that people smoke tobacco in order to enjoy the primary rewarding properties of nicotine, and avoid the adverse consequences of its withdrawal following chronic exposure to the drug. However, several secondary effects of nicotine and tobacco use may also contribute to both the maintenance of smoking and smoking relapse. These include modulation of mood (e.g. reduction of negative affect), stress reduction, antinociception, weight control and cognitive enhancement. In addition, condition cues can elicit the urge to smoke, even after prolonged periods of abstinence.

Pharmacological therapies for smoking cessation fall into two major categories. The first is nicotine replacement therapy (NRT), which enables smokers to substitute the nicotine from cigarettes with other nicotine formulations. The second category is non-nicotine based, and the success of the atypical antidepressant bupropion (Zyban®) as an anti-smoking agent, has raised much debate as to how a non-nicotine based drug can aid in smoking cessation. This section will summarise various medications currently available in the market, or have the potential to aid smoking cessation.

1.5.1 Nicotine replacement therapy

1.5.1.1 Existing NRTs

NRT is the most commonly used type of medication to treat nicotine dependence in the USA and other countries. At present, there are five types of NRTs, which include the slow-acting transdermal nicotine patch, and faster acting formulations such as nicotine gum, nicotine nasal spray, nicotine vapour inhaler and, most recently, the nicotine lozenge. The faster-acting NRTs appear to help satiate the positive effects of nicotine administration achieved through smoking, and reduce acute craving. The slow-acting nicotine patch, however, supplies constant, low levels of nicotine which, when adequately dosed, relieves nicotine-withdrawal symptoms (Foulds et al., 2004; George and O'Malley, 2004).

Although NRTs may have the capacity to provide nicotine-mediated neuropharmacological effects, some of these formulations have their drawbacks. For example, nicotine gum is often not used properly and is not effective across all settings. Similarly, nicotine nasal spray is aversive for many smokers, and adherence rates tend to be lower than those for other forms of NRT (Berrettini and Lerman, 2005). Furthermore, all NRTs are perceived by smokers as being markedly less satisfying than cigarettes and none of them deliver the same quantity of nicotine to the brain with the speed of a cigarette (Foulds et al., 2004). As a result, none of the NRTs enable the majority of people who use them, to quit smoking in the long term, as up to 80% of those who try to quit using these aids, relapse within a year (Cryan et al., 2003b).

1.5.1.2 Potential new NRTs

Nicotine straw

This is a single-use drinking straw that contains a loose bead formulation of nicotine bitartrate. It has a fluid permeable plug at the bottom that facilitates the ingestion of nicotine in the product with the first sip of a beverage (Foulds et al., 2004). The hand-to-mouth motion makes the Straw user-friendly to smokers who are trying to quit. Presently, the Straw is being tested in Phase III clinical trails and preliminary data suggests that it is well-tolerated, with some minor side-effects such as nausea and light-headedness. The Straw, however, does not provide improved speed of nicotine delivery over other NRTs. Also, it may carry risks associated with misuse and potential overdose (Foulds et al., 2004).

Nicotine solution

Nicotine solution drops are another oral delivery method for nicotine replacement. These are also presently being examined in Phase III clinical trials. Subjects tested have reported that they are well-tolerated with a low quit rate at the 6-month follow-up point. The advantage of the nicotine solution is that its dose can be finely adjusted to the treatment intensities of nicotine dependence. Furthermore, the slow absorption rate may not only reduce the dependence potential, but also minimise the use of the solution as an acute craving rescue medication (Foulds et al., 2004).

1.5.2 Non-nicotine based treatments

1.5.2.1 Antidepressants

There is good rationale for testing antidepressants for smoking cessation, as there is a strong correlation between smoking and depression. For example, a high prevalence of smoking exists among people who have, or have had a history of, depression (Covey, 1999). Furthermore, smokers who are depressed find it more difficult to quit smoking, some smokers become depressed when they stop smoking, and post-cessation depression is related to relapse (Covey, 1999; Covey et al., 2000).

Bupropion

Bupropion (Fig. 1.21), an atypical antidepressant, is the first non-nicotine medication to demonstrate efficacy in the treatment of tobacco dependence. Although the precise mechanism by which bupropion aids smoking cessation remains uncertain (see section 1.6), it has proven to increase abstinence rates (Richmond and Zwar, 2003) and reduce severity of withdrawal symptoms experienced by smokers when they abstain (Hurt et al., 1997; Jorenby et al., 1999; Shiffman et al., 2000; Hays et al., 2001; Durcan et al., 2002).

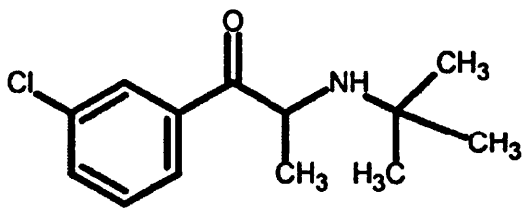


Fig. 1.21 The chemical structure of bupropion.
Bupropion has a molecular weight of 239.7 Daltons. Although initially used as an antidepressant (Wellbutrin), it is now marketed under the commercial name of Zyban®, as a non-nicotine aid to smoking cessation.

With the exception of two recent clinical trials (Killen et al., 2004; Simon et al., 2004), several others have demonstrated the relative efficacy of bupropion as a smoking cessation aid. Amongst these were two pivotal outcome studies published in the late 1990s (Hurt et al., 1997; Jorenby et al., 1999). The first was a randomised, double blind trial that compared the effects of three different daily doses of bupropion (100, 150 and

300 mg) with placebo, on a variety of measures including smoking abstinence, symptoms of depression and withdrawal, and body weight (Hurt et al., 1997). Subjects were asked to take bupropion or placebo for seven weeks, beginning one week before complete cessation of cigarette smoking. Smokers with current depression, but not those with a history of depression were excluded. Results showed a linear effect on point-prevalence cessation, although no significant difference was found between 150 and 300 mg/day bupropion at 12 months follow-up. Additionally, there was no change in depression scores for any treatment group, suggesting that the participants did not experience significant depressive symptoms during withdrawal. Also, subjects who had quit smoking once treatment had ceased showed attenuated weight gain. The second major study was also double-blind and randomised smokers to receive either bupropion alone (300 mg/day), nicotine patch alone (21 mg/21 h), both bupropion and patch, or placebo (Jorenby et al., 1999). The recording measures and exclusion criteria were largely similar to those used by Hurt and colleagues (1997). The one-year continuous abstinence rates were 18%, 10%, 23% and 6% in the bupropion alone, patch alone, combination-treatment, and placebo groups, respectively. Results of the withdrawal and depression scores were similar to those observed by Hurt and colleagues (1997). Jorenby and colleagues (1999) concluded that, although bupropion was significantly better than the nicotine patch, abstinence rates in the combination regime were not significantly different from bupropion alone.

Since these clinical trails, a number of other studies examining the efficacy of bupropion have been published, mainly corroborating the findings of the original trials (Johnston et al., 2001; Stahl et al., 2004). They confirm its efficacy among patients who may be considered difficult populations to treat, because of their illnesses. In particular, bupropion has been shown to assist those with cardiovascular disease (Tonstad et al., 2003) and chronic obstructive pulmonary disease (Tashkin et al., 2001), to quit smoking with cessation rates equivalent to healthy populations. In addition, bupropion proved efficacious for smoking cessation in patients independent of a former history of major depression or alcoholism (Hayford et al., 1999), indicating that the success of bupropion is not related to its antidepressant effect. Further trials have achieved positive results for the use of bupropion in preventing relapse after initial treatment (Hays et al., 2001; Wileyto et al., 2005), and for effective smoking cessation when combined with minimal or moderate counselling (Swan et al., 2003). In studies regarding nicotine craving and withdrawal, bupropion was found to alleviate some symptoms of nicotine withdrawal but had no effect on craving for cigarettes (Shiffman et al., 2000). In contrast, another study showed that bupropion contributed to preventing relapse by reducing craving after the initial withdrawal from nicotine (Durcan et al., 2002). In non-treatment-seeking smokers, acute doses of bupropion has shown to increase smoking without altering rates of craving or withdrawal, whilst under controlled conditions, bupropion helped to reduce smoking in patients willing

to quit (Cousins et al., 2001). Recent trials have failed to provide confirmatory findings on the clinical efficacy of bupropion. For example, although a reduction in cigarette consumption was noted, a randomised trial in adolescent smokers failed to observe a treatment effect of bupropion when combined with the nicotine patch (Killen et al., 2004). Similarly, a seven-week course of bupropion in combination with cognitive-behavioural counselling and NRT failed to demonstrate a sustained benefit (Simon et al., 2004). Even though the majority of studies have showed bupropion to be an effective aid in smoking cessation, more so than placebo or the nicotine patch, none of the trials have shed much light on how bupropion exerts its clinical efficacy as an anti-smoking agent (see section 1.6.2 for the pharmacological actions of bupropion).

Bupropion is marketed for smoking cessation under its trade name Zyban. So far, no gender- or age-related differences in pharmacokinetics of bupropion have been observed (Johnston et al., 2002). Based on this knowledge, the target dose of this agent in nicotine dependence is 300 mg/day. It is started seven days before the target quit date at 150 mg/day, and is increased to 150 mg/day twice daily after three days so that steady-state levels are achieved before the quit attempt. The recommended duration of treatment is between 7-9 weeks in Europe and Australia, and 7-12 weeks in USA and Canada. For the best chance of success, bupropion should be used in conjunction with regular behavioural counselling. If the drug is not efficacious in helping the patient to quit smoking, it should be terminated after seven weeks of use (Richmond and Zwar, 2003). The primary side-effects associated with bupropion are headache, jitteriness, dry mouth, initial insomnia and gastrointestinal problems. Bupropion is contraindicated in patients with seizure disorders and in those with bulimia or anorexia nervosa, or a history of these disorders (Holm and Spencer, 2000).

Nortriptyline

Nortrptyline, a tricyclic antidepressant (TCA), is believed to aid smoking cessation via its ability to block the reuptake of NA (and possibly 5-HT), and thereby reduce withdrawal and depressive symptoms (Covey et al., 2000; Foulds et al., 2004). The database for nortriptyline is not as extensive as that for some NRTs or bupropion. So far six randomised-controlled trials have been conducted for the efficacy of nortriptyline as an anti-smoking agent (Hughes et al., 2005). Pooled results from the studies that compared nortriptyline with placebo, found nortriptyline to at least double the chance of long-term abstinence. In contrast, when nortriptyline was tested as an add-on to nicotine patch treatment, it produced a significant benefit in one study but not in the other (Hughes et al., 2005). The side-effects of nortrptyline include dry mouth, light-headedness, shakiness, blurred vision, urinary retention, constipation, and sexual difficulties. Nortriptyline is contraindicated in those who have experienced recent myocardial infarction or arrhythmias, or have a history of epilepsy or psychosis, liver disease, or women who are

pregnant or breast-feeding (Covey et al., 2000; Hughes et al., 2005). Despite the evidence of efficacy and relatively low cost, nortriptyline has not obtained regulatory approval for treating tobacco dependence nor been marketed for this indication (Foulds et al., 2004).

Reboxetine

Nicotine increases cortical NA in rats (Benwell and Balfour, 1997). Furthermore, locus coeruleus α2-noradrenergic autoreceptors are markedly down-regulated in smokers (Klimek et al., 2001), suggesting that nicotine-induced NA release may result in adaptive processes in feedback mechanisms that regulate NA function. A recent animal study shows that the antidepressant reboxetine, a selective NA reuptake inhibitor, dose-dependently decreases nicotine self-administration in rats (Rauhut et al., 2002). Its effects may be facilitated by its ability to inhibit nAChR function or by indirectly modulating the firing pattern of DA cells in the VTA and selectively increasing DA availability in the PFC (Cryan et al., 2003b). Hence, there is reason to suspect that reboxetine may have the potential be an effective tobacco-dependence treatment.

Monoamine oxidase inhibitors

Monoamine oxidase (MAO) inhibitors degrade monoamine neurotransmitters. MAO-A preferentially degrades 5-HT and NA, and MAO-B selectively metabolises DA (George and O'Malley, 2004). Smoking is known to inhibit MAO-A and MAO-B (Covey et al., 2000). Thus, MAO inhibitors such as selegiline, lazabemide and moclobemide, may aid smoking cessation through their ability to inhibit DA metabolism.

Selegiline, a selective MAO-B inhibitor and an indirect DA agonist, is used to treat Parkinson's disease. A number of small randomised-controlled trials, comparing selegiline to placebo or nicotine patch, have suggested that salegiline may alleviate withdrawal symptoms and reduce craving for nicotine (Foulds et al., 2004).

Moclobemide is a reversible MAO-A inhibitor that is available in Europe and Canada to treat major depression (George and O'Malley, 2004). It has been examined in one long-term trial, which found a significant effect on smoking cessation at 6 months, but not 1 year, compared to placebo (Berlin et al., 1995).

Recently, an exploratory trial on lazabemide, a reversible MAO-B inhibitor, found that it increased short-term abstinence to smoking compared to placebo (Berlin et al., 2002). However, the study had to be terminated prematurely, as there were reports of liver toxicity from studies investigating this drug for other purposes.

Other antidepressants

Trials of other antidepressants as smoking cessation aids have reported with generally promising but as yet inconclusive results. One study suggested that doxepin, a

TCA, reduced urges to smoke, but failed to show a significant effect on abstinence (Covey et al., 2000). A few studies have examined the efficacy of selective serotonin reuptake inhibitors (SSRI), fluoxetine and paroxetine, in smoking cessation. One study showed a significant short-term effect of fluoxetine on abstinence in smokers with a history of major depression (George and O'Malley, 2004). However, none of the other studies reported any long-term benefit with either SSRI (Covey et al., 2000).

1.5.2.2 nAChR ligands

Varenicline

Verenicline (CP-526555; Pfizer) is presently in Phase III clinical trials as a smoking cessation aid. It is an active $\alpha4\beta2$ nAChR partial agonist and exhibits *in vivo* properties that demonstrate its ability to attenuate the central dopaminergic response to nicotine while providing sufficient and sustained dopaminergic tone to limit craving and withdrawal (Coe et al., 2005). Furthermore, it is not believed to be addictive (Foulds et al., 2004). Varenicline was developed from the structural starting point provided by cytisine, which is also a partial agonist of $\alpha4\beta2$ nAChRs (Coe et al., 2005). Cytisine has a mechanism of action similar to that of nicotine but with rather low toxicity and short-lived effects. As of yet, no clinical studies have been published of varenicline, but research is underway comparing varenicline with bupropion (Foulds et al., 2004).

Mecamylamine

Mecamylamine is a potent non-competitive nAChR antagonist. The rationale for using mecamylamine to aid smoking cessation is that it should reduce the satisfaction associated with smoking and the urge to smoke. When this drug was given to smokers, cigarette consumption increased in an attempt to overcome nAChR blockade (George and O'Malley, 2004). Early attempts to use mecamylamine alone for smoking cessation were unsuccessful as it also induced side-effects such as abdominal pain, constipation, dry mouth and headaches. However, recent studies have suggested that mecamylamine in combination with nicotine patch produces a superior outcome compared to nicotine patch and placebo (Covey et al., 2000). The success is owed to the belief that nicotine (delivered by an alternative route than cigarette smoking) and mecamylamine would work in concert to attenuate the rewarding effects of cigarette smoking, suppress withdrawal symptoms, and thereby facilitate smoking abstinence. The combination treatment of mecamylamine and nicotine patch is presently in Phase III clinical trials as a smoking cessation aid.

1.5.2.3 Cannabinoid receptor antagonist

It has been hypothesised that drugs affecting cannabinoid mechanisms may have a role in smoking cessation. Drugs that selectively block cannabinoid-1 (CB1) receptors are believed to modulate energy balance systems in the brain, which are thought to be altered in smokers (Foulds et al., 2004). Preclinical pharmacological studies show that rimonabant (SR-141716-A), a CB1 receptor antagonist, reduces nicotine self-administration (Cohen et al., 2005), nicotine-conditioned place preference (Le Foll and Goldberg, 2004) and nicotine-induced DA release in the NAc (Cohen et al., 2005). Rimonabant also abolishes the maintenance of nicotine seeking behaviour induced by cues associated with nicotine that are crucial for nicotine craving and relapse (Le Foll and Goldberg, 2005). Although studies using knockout mice have attempted to clarify the involvement of CB1 receptors in nicotine rewarding properties, the results obtained have provided conflicting data. However, pharmacological data suggest that rimonabant could have anti-smoking activity. Accordingly, it is presently being tested in placebo-controlled Phase III trials (Cryan et al., 2003b).

1.5.2.4 DA D3 receptor antagonist

In contrast with D1 and D2, D3 receptors have a restricted expression pattern in the brain, being selectively expressed in the NAc_{shell} (Cryan et al., 2003b). Chronic administration of nicotine is associated with marked increases in D3 receptor binding and mRNA expression in the NAc_{shell} (Le Foll et al., 2003). The selective D3 receptor antagonist, SB-277011-A, is efficacious in animal models of nicotine-seeking behaviour (Heidbreder and Hagan, 2005) and awaits clinical validation for prevention of relapse to smoking in humans.

1.5.2.5 α2-adrenoceptor agonist

Clonidine is an α2-adrenoceptor agonist that reduces sympathetic activity by decreasing NA release. It was originally approved for its use as an antihypertensive and for the prevention of recurrent migraine, but has recently found to be effective in the treatment of opioid and alcohol dependence (George and O'Malley, 2004). Several clinical trials have demonstrated that clonidine taken orally or as a transdermal patch, has modest efficacy in smoking cessation. However, significant side-effects such as postural hypotension, sedation, constipation, dizziness and dry mouth, have limited its use. Thus clonidine is considered a second-line pharmacotherapy for smoking cessation (George and O'Malley, 2004).

1.5.2.6 Opioid antagonists

Opioid antagonists, such as naloxone and its longer-acting formulation naltrexone, are used in the treatment of other chemical dependencies, such as opioid, cocaine and alcohol. The rationale for using these antagonists in treating tobacco dependence comes from the principle that endogenous opioids may play a role in the reinforcing effects of nicotine (Foulds et al., 2004). A number of studies on the effects of naloxone and naltrexone on smoking behaviour in humans have been conducted, but with mixed results. Some studies with naloxone observed a reduction in cigarette consumption compared with placebo, whilst others failed to demonstrate this effect (Covey et al., 2000). There is little evidence that naltrexone alone facilitates smoking cessation. However, more encouraging results were obtained when naltrexone was combined with nicotine patch (George and O'Malley, 2004) or behavioural counselling (Covey et al., 2000). Increased side-effects, especially sedation, are a common feature of naltrexone treatment. In experimental studies of drugs that have such adverse effects, it is difficult to determine whether the side-effects are making the subjects too sedated or nauseous to smoke or whether the drug is having a direct and substantial therapeutic effect (Foulds et al., 2004).

1.5.2.7 Anxiolytics

Smoking has long been interpreted as a form of "stress management", or a means of reducing anxiety. As a result, a number of efforts had been made to investigate the potential efficacy of anti-anxiety drugs as an aid to smoking cessation. Studies with traditional sedatives, such as barbiturates and benzodiazepines, and other sedative-like drugs, such as β-blockers, have not been found to aid smoking cessation (Covey et al., 2000). On the contrary, buspirone, an atypical anxiolytic, has attracted some interest. Buspirone is a selective 5-HT1A receptor agonist, with mild D2 receptor activity. It is prescribed for the management of anxiety disorders, but is pharmacologically unrelated to barbiturates and benzodiazepines, and so is non-sedative and non-addictive. Early un-controlled studies indicated that buspirone decreased nicotine withdrawal symptoms and reduced the urge to smoke, whilst recent long-term studies comparing buspirone with placebo showed no benefit of using buspirone for smoking cessation. However, buspirone has been shown to enhance short-term smoking cessation rates only in those smokers who are anxious (Covey et al., 2000). Hence, buspirone may be helpful for smokers with a high level of anxiety, but there is insufficient evidence to support the general usefulness of buspirone as a smoking cessation aid.

1.5.2.8 Oral dextrose

The idea of oral dextrose (glucose) for smoking cessation is based on the premise that the urge to smoke arises in part from a mislabelling of a physiological "desire" for carbohydrates (Covey et al., 2000). Its advantage is that it is relatively inexpensive and may be a safer alternative to other tobacco-dependent medications. Placebo-controlled studies have provided some support for the idea that glucose relives craving for carbohydrate and hence overall craving (Foulds et al., 2004). Not all studies using glucose alone have yielded positive results on smoking cessation. However, preliminary data from a randomised placebo-controlled trial suggests that glucose tablets increase quit rates compared to placebo among patients using concurrent bupropion or NRT (Foulds et al., 2004).

1.5.2.9 Nicotine vaccine

After the introduction of vaccines against cocaine (Kosten and Owens, 2005), efforts to develop a nicotine vaccine soon followed. The principle behind vaccination is that a small amount of a substance (antigen) is injected into a host. The host's immune system will recognise this antigen as foreign and subsequently produce antibodies against it (immune response). Since nicotine is a small molecule, it is bound to a larger molecule such as bovine serum albumin (BSA) or inactivated bacterial toxins, which increase its antigenicity. This is likely to augment the probability of an immune response by the host. After the initial vaccination, the host immune system memorises the antigen such that if it was introduced in the future, the immune cells would be able to produce large amounts of antibodies, which would bind to the antigens and allow for other host defences to eliminate them. The blood-brain barrier (BBB) acts as a filter and prevents molecules that are too large to pass through. The nicotine molecule is small enough to pass into the brain. However, as a result of vaccination, the nicotine-antibody complex becomes too large to pass through the BBB. Therefore, vaccination would reduce or eliminate the effects of nicotine in the brain. This would in theory, prevent the psychoactive effects of nicotine by reducing the rewarding effects of smoking and drive for continued consumption (Foulds et al., 2004).

In rats, the nicotine vaccine elicits antibodies that reduce nicotine distribution to the brain by 40-60% (Kosten and Owens, 2005). Immunisation also decreases nicotine-induced DA release, acquisition of nicotine self-administration, and nicotine seeking behaviour in animal models of relapse (Kosten and Owens, 2005).

Three nicotine vaccines are presently undergoing human testing. NicVAX (Nabi Biopharmaceuticals), which is presently being tested in Phase II trials, consists of nicotine

conjugated to a carrier protein called recombinant exoprotein A. Phase I trails showed that a single dose of NicVAX produced antibodies as early as 7 days post-vaccination and these were maintained over 4 months (Foulds et al., 2004). Nicotine-Qbeta (Cytos Biotechnology), which is also currently being tested in Phase II trials, uses antigens delivered in a repetitive configuration that can directly activate B cells. A double-blind placebo-controlled trail for nicotine addiction showed that 57% of patients with a high antibody response to Nicotine-Qbeta met the primary end-point of continuous abstinence (Kosten and Owens, 2005). Finally, TA-NIC (Xenova Research Ltd) comprises nicotine conjugated to a carrier protein and an adjuvant, and so designed to induce nicotine antibodies that would bind free nicotine in the blood making the nicotine-antibody complex unable to cross the BBB (Foulds et al., 2004). TA-NIC is presently being tested in Phase I clinical trials. Preliminary data suggest that this vaccine is safe and well tolerated but there is a 20-fold variation in individual patient responses (Kosten and Owens, 2005).

The nicotine vaccine approach raises issues that still need to be addressed. These include the lack of protection against a structurally dissimilar molecule that produces the same effects as nicotine, as well as the individual variability in antibody formation. Furthermore, vaccination in animals shows the highest titres of antibody in serum, which highlights the importance of producing high antibody titres in humans to avoid overcoming antibody therapy to aid vaccine efficacy. Vaccination is not expected to show efficacy against nicotine craving or withdrawal, and so may be used in conjunction with other current medications (Heidbreder and Hagan, 2005).

1.5.2.10 Other non-nicotinic pharamcotherapies

Although both GABA and glutamate systems have been implicated in nicotine addiction, largely because of their close interactions with the mesolimbic DA neurones, few clinical studies have tested their utility in the treatment of nicotine dependence. A human laboratory study with the GABA-B receptor agonist, baclofen, indicated that it could reduce the rewarding effects of smoking (George and O'Malley, 2004). However, one of the drawbacks of baclofen therapy is the marked muscle relaxation and sedative properties of the drug, which hamper its widespread use in indications outside of that as an antispastic medication (Cryan et al., 2003b). Preclinical data suggest a role for presynaptic metabotropic glutamate receptors (mGluR) in nicotine dependence. For example, blockade of mGluR-5 with 6-methyl-2-(phenylethynyl)-pyridine decreases the reinforcing effects of nicotine, whilst blockade of mGluR-2/3 with LY-354740 reverses the affective signs of nicotine withdrawal (Cryan et al., 2003b). Some of these agents look promising, but require further evidence before becoming generally recognised effective treatments for smokers.

1.6 Bupropion

Despite bupropion having been in clinical use for 25 years, the neurochemical mechanisms underlying its actions as a smoking cessation aid are still not well-elucidated (Ascher et al., 1995). Various preclinical studies have suggested that bupropion produces its therapeutic antidepressant effects by blocking DA and NA reuptake (Ascher et al., 1995). As these neurotransmitters have a pivotal role in the rewarding properties of nicotine and the aversive state of nicotine withdrawal, the antidepressant properties of bupropion may be significant in smoking cessation. Recent *in vitro* data indicate that bupropion may be a noncompetitive, functional inhibitor of neuronal nAChRs (Fryer and Lukas, 1999; Slemmer et al., 2000). This anti-nicotinic activity of bupropion could act to acutely attenuate the rewarding effects of nicotine, thus increasing the likelihood of smoking cessation. Bupropion's mode of action is complicated by differences in its *in vitro* vs. *in vivo* pharmacological profile (Richelson and Pfenning, 1984; Stathis et al., 1995) along with interspecies variations in the metabolism of bupropion (Suckow et al., 1986; Tatsumi et al., 1997). Hence, this section will summarise previous evidence from preclinical studies to facilitate the understanding of bupropion's mechanisms of activity in encouraging smoking abstinence.

1.6.1 Pharmacokinetics of bupropion

Bupropion (Fig. 1.21) is an aminoketone that is highly lipophilic, enabling the drug to enter many tissues, including the brain. In healthy humans, peak concentration (C_{max}) of approximately 136 µg/l in the plasma, is achieved within 3 h after an oral dose of 150 mg bupropion (Hsyu et al., 1997). The volume of distribution estimated from a single 150 mg dose is approximately 2000 l (Johnston et al., 2002). Plasma protein binding of bupropion is approximately 85% over a wide range of concentrations in humans, and only a little less in three other species (75-80%; Schroeder, 1983).

Bupropion is extensively metabolised by multiple pathways to three active metabolites: hydroxybupropion, which is formed via hydroxylation of the tert-butyl group and the amino alcohol isomers; threohydrobupropion and erythrohydrobupropion, which are formed by the reduction of the carbonyl group (Fig. 1.22; Johnston et al., 2002). Bupropion and the metabolites undergo further biotransformation and conjugation to form meta-chlorohippuric acid, which is the major urinary metabolite (Holm and Spencer, 2000). *In vitro* studies in human liver microsomes have shown that the conversion of bupropion to hydroxybupropion is catalysed primarily by the CYP 2B6 and to a much lesser extent by CYP 1A2, 2A6, 2C9, 2E1, and 3A4 isoenzymes (Johnston et al., 2002).

The formation of threohydrobupropion or erythrohydrobupropion does not involve CYP isoenzymes.

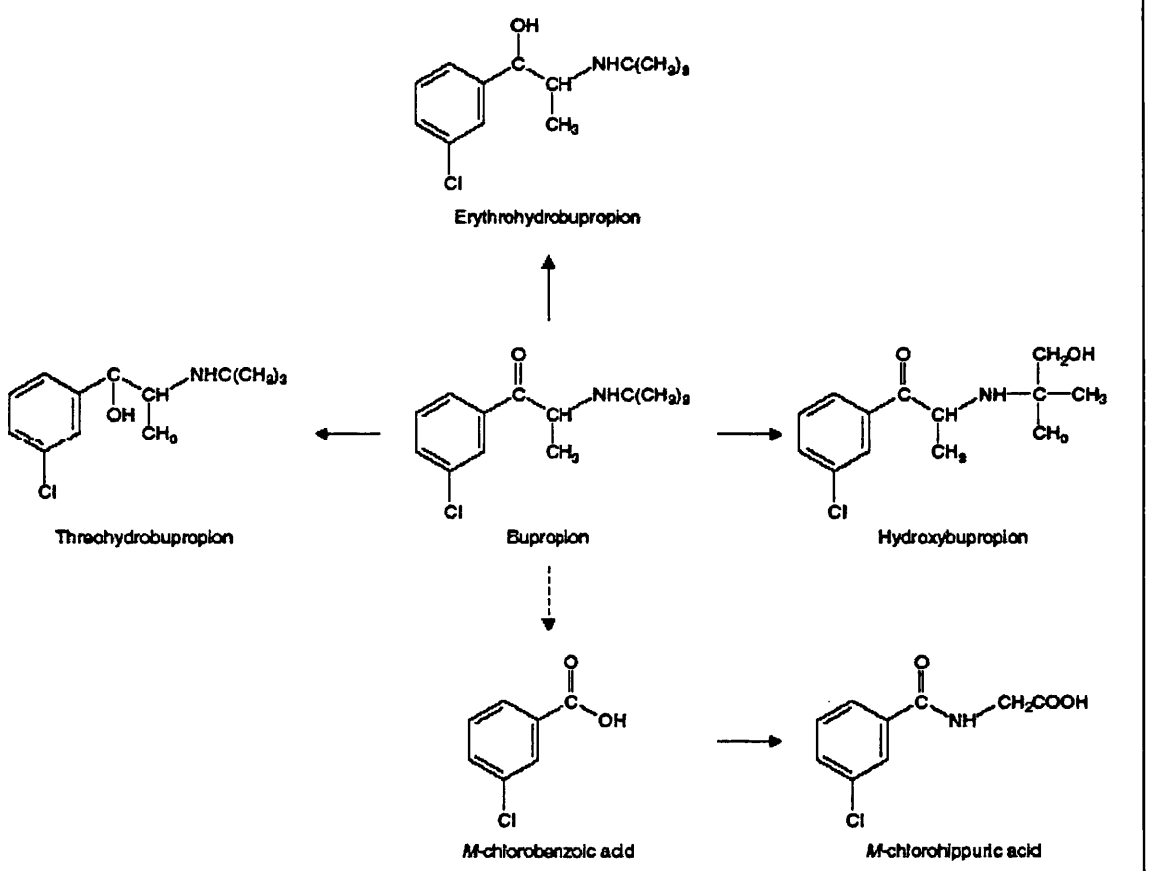


Fig. 1.22 The major pathways of bupropion metabolism in humans.
(adapted from Hsyu et al., 1997).

The potency of hydroxybupropion is comparable to that of bupropion, whilst the other metabolites threohydrobupropion and erythrohydrobupropion are one-fifth as potent as bupropion (Johnston et al., 2002). After the administration of a single 150 mg dose of bupropion in humans, area under the concentration curve (AUC), C_{max}, and time to reach C_{max} (t_{max}) values in plasma for hydroxybupropion are considerably greater than those of the parent compound (Table 2a; Hsyu et al., 1997; Holm and Spencer, 2000). Combined, the metabolites threohydrobupropion and erythrohydrobupropion also have a greater plasma AUC value, and longer t_{max} and $t_{½}e$ than bupropion (Hsyu et al., 1997). A similar pharmacokinetic pattern is seen in mouse plasma and brain after a systemic injection of 40 mg/kg bupropion, whilst rats form little of the metabolites as compared to mice (Table 2b; Suckow et al., 1986).

In a study using radiolabelled bupropion in healthy humans, 87% and 10% of bupropion was recovered, primarily as metabolites, in the urine and faeces, respectively. The fraction of the radioactivity excreted in the urine as unchanged bupropion was only 0.5%, whilst less than 10% of the dose was accounted for in the urine as active metabolites. The remaining radioactivity in the urine was associated with at least nine

other metabolites, some of which have not been fully characterised. Overall, this study shows that: (1) essentially all of the dose could be accounted for in the urine or faeces; (2) bupropion is extensively metabolised; (3) renal elimination is a more important pathway for the removal of the inactive metabolites of bupropion than the active metabolites (Johnston et al., 2002).

a

		$AUC_{0-\infty}$ (µg/l/h)	C_{max} (µg/l)	t_{max} (h)	$t_{½}e$ (h)	Cl (l/h)
Bupropion	Smokers	1164	144	3.0	19	133
	Non-smokers	1161	143	2.9	18	138
Hydroxybupropion	Smokers	15239	430	6.2	22	
	Non-smokers	16651	433	7.7	23	
Threohydrobupropion + Erythrohydrobupropion	Smokers	5698	135	5.5	48	
	Non-smokers	6685	150	4.9	47	

b

			$AUC_{0-\infty}$ (µg/ml/h)	C_{max} (µg/ml)	t_{max} (h)	$t_{½}e$ (h)	Cl (l/h/kg)
Bupropion	Rat	Plasma	3.22	3.85	0.26	0.83	12.41
		Brain	30.13	36.51	0.26	0.79	
	Mouse	Plasma	1.71	2.15	0.26	1.18	23.44
		Brain	16.48	20.66	0.26	0.65	
Hydroxybupropion	Rat	Plasma	0.42	0.20	0.68	0.79	
		Brain	2.58	1.14	0.68	0.71	
	Mouse	Plasma	7.96	2.13	0.39	2.44	
		Brain	58.57	20.22	0.50	1.71	
Threohydrobupropion	Rat	Plasma	0.15	0.07	0.46	0.92	
		Brain	2.36	1.17	0.60	0.68	
	Mouse	Plasma	0.88	0.14	0.26	5.17	
		Brain	9.47	1.99	1.15	2.49	

Table 2 Summary of pharmacokinetic parameters for bupropion and its metabolites.
(a) Pharmacokinetics of bupropion and its major metabolites in plasma after a single oral 150 mg dose in humans.
(b) Pharmacokinetics of bupropion and its major metabolites after a systemic injection of bupropion (40 mg/kg, i.p) in rats or mice.
$AUC_{0-\infty}$, area under the concentration curve from time 0 extrapolated to infinity; C_{max}, peak concentration; t_{max}, time to reach peak concentration; $t_{½}e$, elimination half-life; Cl, clearance (adapted from Suckow et al., 1986; Holm and Spencer, 2000).

1.6.2 Pharmacodynamics of bupropion

1.6.2.1 Bupropion and dopaminergic systems

Studies from rat synaptosomes show that bupropion is twice as potent at inhibiting DA (K_i = 0.6 µM) than NA reuptake (K_i = 1.5-2.3 µM), and has little influence on 5-HT reuptake (K_i = 15.6-19.0 µM; Hyttel, 1982; Richelson and Pfenning, 1984). Lesion studies reveal a preferential affinity of bupropion for DAT in the brain, since it selectively

antagonises DA (ID_{50} = 54 mg/kg, i.p) but not NA depletion (ID_{50} > 100 mg/kg, i.p) induced by 6-hydroxydopamine (Cooper et al., 1980; Ferris et al., 1982; Ascher et al., 1995), which depletes DA and NA only after being taken into DA- or NA-releasing cells via the respective transporters. Bupropion also suppresses activity of midbrain DA cells (ID_{50} = 42-44 mg/kg, i.p; Cooper et al., 1994). However, bupropion doses used in those studies appear to be far higher than those that produce antidepressant effects (Ascher et al., 1995). As doses of bupropion prescribed for smoking cessation are equal to or lower than those prescribed for depression, bupropion levels required to antagonise DA reuptake will also bear little resemblance to those effective in smoking cessation (Warner and Shoaib, 2005). However, the antidepressant effects of bupropion, as measured by immobility in the forced swim test, are prevented by prior destruction of DA neurones by 6-hydroxydopamine (Cooper et al., 1980), indicating that the antidepressant actions of bupropion, at least, are dependent on dopaminergic activity.

In vivo brain microdialysis studies reveal that bupropion-mediated effects on DA neurotransmission vary according to different areas of the brain and on the duration of treatment (acute vs. chronic). Local application of bupropion into the striatum dose-dependently increases striatal extracellular DA levels (Nomikos et al., 1990). Increased levels of extracellular DA are also seen in the striatum and NAc after acute systemic bupropion (Nomikos et al., 1989). Furthermore, these bupropion-induced increases in extracellular DA are blocked by TTX, indicating that its effects rely on action potential-dependent processes in DA neurones (Nomikos et al., 1989; Nomikos et al., 1990). Chronic treatment with bupropion significantly enhances the increase in extracellular DA in the NAc, when rats are challenged with systemic bupropion after a 3 day withdrawal period (Nomikos et al., 1992). This apparent sensitised effect is not seen in the striatum (Nomikos et al., 1992), thereby highlighting a regional selectivity for the effects of bupropion after chronic treatment. Furthermore, as these effects of bupropion occur at doses that have been associated with antidepressant activity in animal models, it suggests that the relatively weak DA reuptake antagonism by bupropion can still affect the dopaminergic system primarily implicated in nicotine dependence (Ascher et al., 1995; Warner and Shoaib, 2005).

In behavioural studies that measure locomotor activity, acute systemic administration of bupropion dose-dependently increases hyperlocomotion (Cooper et al., 1980; Nielsen et al., 1986). However, rats with depleted DA neural systems fail to display significant locomotor stimulation after bupropion treatment (Cooper et al., 1980), thus demonstrating that motor activity is a DA-mediated behaviour that requires intact DA neurones. Chronic administration of bupropion has shown to result in augmented locomotor activity after a systemic bupropion challenge (Cooper et al., 1980; Nielsen et al., 1986). This sensitised effect is comparable to the enhanced increase in extracellular DA in the NAc following chronic bupropion treatment (Nomikos et al., 1992). Bupropion-

induced sensitisation could be attributed to an upregulation of DAT, which is seen in the NAc after chronic bupropion self-administration (Tella et al., 1997). In a recent study, locomotor-stimulating effects of bupropion were observed at different ages, with late adolescent mice being more active than early adolescent or adult mice (Redolat et al., 2005). This is pertinent in the use of bupropion to combat nicotine dependence, as differential sensitivity to nicotine has also been reported to be age-dependent (Belluzzi et al., 2004).

A positron emission tomography study that examined the occupancy of DAT showed that there is a real possibility of bupropion having an effect on DA reuptake during clinical treatment in humans. By measuring the displacement of a selective and reversible DAT-binding radioligand (^{11}C-βCIT-FE), chronic bupropion was found to occupy an average of 26% of striatal DAT over a period of 24 h at steady-state oral dosing in non-depressed humans (Learned-Coughlin et al., 2003). The relationship between the level of DAT occupancy and the therapeutic effects of bupropion is unknown. However, to exert an effect on DA reuptake, DAT must be implicated in some way to bupropion's mechanism of action (Warner and Shoaib, 2005).

If bupropion is capable of maintaining raised extracellular DA levels in brain regions such as the NAc, it could reduce the deficit of DA overflow and brain reward function caused by nicotine withdrawal. Therefore, it is possible that bupropion may be efficacious in smoking cessation by ameliorating some of the negative affective aspects of nicotine withdrawal, and thereby help to prevent relapse.

1.6.2.2 Bupropion and noradrenergic systems

The potency of bupropion as an inhibitor of NA reuptake is only half of that for DA (see section 1.6.2.1). This may not have a fundamental role in the efficacy of bupropion as a smoking cessation aid, but there is a small body of evidence that implicates noradrenergic effects in the mechanism of action of bupropion.

At high doses, bupropion significantly decreases NA-stimulated adenylate cyclase activity (Ascher et al., 1995). However, it is believed that this effect occurs at doses of bupropion that are far greater than those that demonstrate antidepressant activity in animal models. *In vivo*, acute administration of bupropion inhibits firing rates of NA neurones in the locus coeruleus at doses relevant to antidepressant activity (ID_{50} = 12.6 mg/kg, i.p; Cooper et al., 1994). However, bupropion-induced inhibition of NA reuptake is thought to be an indirect effect of elevated NA levels in the synaptic cleft, stemming from the finding that yohimbine, an α2-adrenoceptor antagonist, blocks bupropion-induced inhibition of firing rates (Cooper et al., 1994). At clinically relevant doses in man, bupropion reduces whole-body turnover of NA without altering plasma NA levels, indicating that noradrenergic systems are made more efficient (Johnston et al., 2002).

Bupropion appears to have some significant effects on noradrenergic systems, although, these effects probably contribute to the antidepressant activity of bupropion.

1.6.2.3 Bupropion and nAChR antagonism

There is substantial evidence to support the theory that bupropion can antagonise the effects of nicotine at nAChRs. The first evidence came from a study by Fryer and Lukas (1999), where they examined the acute functional effects of bupropion on human nAChR subtypes expressed in cell lines TE671/RD (muscle-type $\alpha1\beta1\gamma\delta$ nAChR) and SH-SY5Y cells (ganglionic $\alpha3\beta4\alpha5 \pm \beta2$ nAChRs), using the $^{86}Rb^{+}$ efflux assay. Bupropion was found to produce dose-dependent inhibition of muscle-type (IC_{50} = 10.5 μM) and ganglionic nAChR (IC_{50} = 1.4 μM) function in TE671/RD and SH-SY5Y cells, respectively (Fryer and Lukas, 1999). Furthermore, the functional block produced by bupropion near the IC_{50} value was insurmountable by increasing agonist concentration in both cell lines, indicating non-competitive inhibition of nAChR function by bupropion (Fryer and Lukas, 1999).

Another study examined the acute interaction of bupropion with nAChRs using different *in vivo* and *in vitro* tests. Bupropion was found to produce a dose-dependent blockade of nicotine-induced antinociception, as measured by the tail-flick (ID_{50} = 2.4 mg/kg, s.c) and hot-plate tests (ID_{50} = 8.0 mg/kg, s.c). Bupropion also inhibited nicotine-induced hypothermia (ID_{50} = 8.5 mg/kg, s.c), hypomotility (ID_{50} = 4.0 mg/kg, s.c) and seizure activity (ID_{50} = 4.5 mg/kg, s.c; Slemmer et al., 2000). Together, these *in vivo* tests suggested that bupropion possesses some selectivity for neuronal nAChRs underlying these various nicotinic effects. In *Xenopus* oocytes expressing the $\alpha4\beta2$, $\alpha3\beta2$ and $\alpha7$ nAChR subunit combinations, bupropion antagonised the effects of ACh in a concentration-related manner with IC_{50} values of 8 μM, 1.3 μM and 60 μM bupropion, respectively (Slemmer et al., 2000). The blockade by bupropion was reversible, dissipating after a 5 min washout period, but insurmountable by increasing concentrations of ACh, indicative of non-competitive nAChR blockade. In addition, bupropion failed to displace brain [^{3}H]nicotine binding sites, suggesting that bupropion does not bind to the active site of the nAChR. Also, inhibition of $\alpha4\beta2$ and $\alpha3\beta2$ nAChRs by bupropion exhibited voltage-independent properties, suggesting that bupropion may not be acting as an open channel blocker (Slemmer et al., 2000).

Miller and colleagues (2002) extended the findings that propose bupropion to inhibit nAChRs. Their study used radiolabelled DA and NA in rat striatal and hippocampal slices, respectively, to measure functional neurotransmitter release. To eliminate the interaction of bupropion with DA or NA transporters, nomifensine or desipramine, respectively, were included in the superfusion buffer. Bupropion inhibited both nicotine-

evoked [^{3}H]DA (IC_{50} = 1.27 μM) and [^{3}H]NA overflow (IC_{50} = 0.32 μM) in striatal and hippocampal slices, respectively (Miller et al., 2002). Bupropion had no effect on electrical field stimulation-evoked [^{3}H]overflow in [^{3}H]DA-preloaded striatal slices (Miller et al., 2002), suggesting that its inhibition of nicotine-evoked [^{3}H]DA overflow is specific to nAChRs, and not just a generalised effect on neurotransmitter release.

The evidence that bupropion may act as a nAChR antagonist suggests that its clinical activity in smoking cessation may involve reducing or attenuating the acute effects of nicotine reinforcement prior to cessation, thereby making it easier to abstain. To build a consistent plasma level, bupropion is only taken for a week prior to quit date (see section 1.5.2.1) and so is unlikely to be at a concentration that is able to exert a profound effect on nicotine reinforcement over a short period. This is very important when considering bupropion treatment for chronic smokers as they would have been reinforced to the effects of nicotine in tobacco over many years. However, it is possible that bupropion-induced blockade of nicotinic effects may enable it to prevent relapse in long-term treatment. Therefore, bupropion combined with long-term abstinence, which itself could break the strength of nicotine reinforcement, may assist smokers to recover from single relapse episodes rather than completely relapsing from renewed reinforcing effects (Warner and Shoaib, 2005).

1.6.2.4 Bupropion and models of nicotine reinforcement

Previous findings suggest that bupropion affects extracellular DA in the NAc (Nomikos et al., 1989; Nomikos et al., 1990). Therefore, the actions of bupropion on nicotine reinforcement may be relevant to its mechanism of action in smoking cessation. Intravenous nicotine self-administration is a preferred model of tobacco dependence and various studies have addressed the effects of bupropion on the reinforcing properties of nicotine.

Shoaib and colleagues (2003) used this model to examine the effects of bupropion on nicotine self-administration. The study found that bupropion failed to attenuate nicotine intake at either dose of bupropion (10 and 30 mg/kg, i.p). In fact, the larger dose of bupropion showed a tendency for rats to self-administer more nicotine over the course of the 28 day treatment period (Shoaib et al., 2003). One reason for this increased behaviour may reflect an attempt to overcome the blockade of nicotine reinforcement. Comparable findings have been observed in the clinic, which saw increased cigarette consumption in smokers treated with mecamylamine (see section 1.5.2.2). A more likely explanation for the increased nicotine intake following bupropion pre-treatment arises from the mild stimulant effects of bupropion that may enhance responding maintained by nicotine.

The findings of Shoaib and colleagues (2003) have been replicated in other studies with different emphasis on the results. One study reported that bupropion has a

biphasic dose-response pattern on nicotine self-administration. At bupropion doses tested in the range by Shoaib and colleagues (2003), nicotine self-administration was increased. However, a very high dose of bupropion (78 mg/kg, s.c), which is probably not clinically relevant, was found to decrease nicotine self-administration (Rauhut et al., 2003). Interpretation for this decreased responding for nicotine was attributed to non-specific effects, such as activation of bupropion-induced hyperlocomotion, which was incompatible with performing the lever-press response for nicotine self-administration. Another study found that acute pre-treatment with bupropion (40 mg/kg, i.p) produces a small significant decrease in nicotine self-administration (Bruijnzeel and Markou, 2003).

An important point to consider is that nicotine intake modelled in laboratory rodents does not accurately mimic that of a chronic smoker. For example the 28 day treatment period studied by Shoaib and colleagues (2003) cannot be compared directly to the nicotine intake regime of a chronic smoker. The nicotine self-administration procedure does, however, allow for short repeated sessions of nicotine intake that are separated by longer periods, which enable plasma nicotine levels to return to normal. Therefore, the evidence presented above seems to suggest that bupropion's mechanism of action does not to lie in altering the reinforcing effects of nicotine (Warner and Shoaib, 2005).

1.6.2.5 Bupropion and models of nicotine withdrawal

Nicotine withdrawal is likely to reduce the positive affect of nicotine reward and thereby influence its reinforcing properties. As bupropion has mild dopaminergic effects, the reward pathway is a possible target underlying the clinical efficacy of bupropion. The use of intracranial self-stimulation (ICSS) procedure has provided a reliable behavioural readout, in laboratory rodents, to assess alterations in brain reward after cessation of drug administration (i.e. drug withdrawal). In this paradigm, electrodes are implanted into brain areas to stimulate sites that animals find rewarding. ICSS has previously shown that withdrawal from chronic nicotine induces a dramatic decrease in brain reward function as reflected by elevations in brain reward threshold (Epping-Jordan et al., 1998). This threshold is one of a few operational measures of the affective aspects of the nicotine withdrawal syndrome (Kenny and Markou, 2001).

Cryan and colleagues (2003) are the only group to have examined the effects of bupropion on brain reward function in relation to nicotine or nicotine withdrawal. They found that under baseline conditions, acute bupropion treatment (10-40 mg/kg, i.p) induced a pronounced lowering of brain reward thresholds, suggesting that it has some effects on DA reward systems independent of nicotine. When co-administered with nicotine, bupropion (10, 20 mg/kg, i.p) had no significant effect on nicotine-induced ICSS. Withdrawal from chronic nicotine administration, which was precipitated by the removal of subcutaneous osmotic mini-pumps that delivered 3.16 mg/kg/day of nicotine for 7 days,

significantly elevated brain reward thresholds. Bupropion dose-dependently (10-40 mg/kg, i.p) lowered the reward thresholds of animals that had been pre-treated with nicotine (Cryan et al., 2003a), suggesting that bupropion clearly attenuates deficits in brain reward function during nicotine withdrawal. This provides an important mechanism of action for bupropion in preventing relapse.

In addition to the ICSS work, Cryan and colleagues (2003) also measured the effects of bupropion on the somatic signs of nicotine withdrawal. Unlike in humans, only the visible somatic signs of withdrawal can be measured in animals. This study measured abdominal constrictions (writhes and gasps), facial fasiculations, eyeblinks, and ptosis (Cryan et al., 2003a). Indeed, withdrawal from chronic nicotine administration significantly increased the amount of total somatic signs of abstinence. Bupropion (20, 40 mg/kg, i.p) significantly reversed the expression of total somatic signs at 12 h post-withdrawal. However, when examined for the effects of bupropion on the individual somatic signs at 12 h post-withdrawal, it demonstrated a significant decrease in the amount of abdominal constrictions but not the other signs (Cryan et al., 2003a).

Together these results suggest that the efficacy of bupropion as an aid to smoking cessation may be due to its ability to alter the rewarding aspects of acute nicotine and reverse the negative affective and somatic aspects of the nicotine withdrawal syndrome. Studies that assess the effects of chronic bupropion treatment on brain reward and nicotine withdrawal would provide better comparisons to bupropion treatment in humans.

1.6.2.6 Bupropion and models of nicotine discrimination

Drug discrimination studies allow animals to respond to the strength of subjective effects of a drug, which determine whether it can be distinguished from vehicle. If drug discrimination is strong, then drug generalisation tests can examine whether other compounds can be substituted for the drug, by sharing similar discriminative effect (Warner and Shoaib, 2005). Hence, another mechanism of action for bupropion may be based upon the subjective effects of nicotine.

Various experiments have tested the effects of bupropion on nicotine's discriminative properties (Wiley et al., 2002; Young and Glennon, 2002; Shoaib et al., 2003). As part of the training process, animals learn to discriminate between the effects of nicotine and vehicle. Following either nicotine or vehicle injections, animals are encouraged to make a specific response (e.g. lever press), which leads to the presentation of reward (usually food pellets). Ultimately, in animals that have learnt to discriminate nicotine from vehicle, challenge injections will inform the animal which response to make in order to receive the reward (Warner and Shoaib, 2005). All the studies used similar discriminating training procedures. However, rats tested by Wiley and colleagues (2002) were trained to discriminate 0.4 mg/kg nicotine from vehicle, Young and

Glennon (2002) used 0.6 mg/kg nicotine, and Shoaib and colleagues (2003) used 0.2 mg/kg nicotine. The difference in training dose may affect the relative strengths and specificity of the discriminative effects and nicotine cue, respectively (Warner and Shoaib, 2005).

Two of the three studies conducted a range of generalisation tests, which found bupropion to substitute for nicotine in a dose dependent manner with complete stimulus generalisation observed at 56 mg/kg bupropion (Wiley et al., 2002) or 21 mg/kg bupropion (Young and Glennon, 2002). These findings suggest that bupropion may aid during nicotine abstinence because of its similarity to the subjective effects of nicotine.

Tests of stimulus antagonism revealed that bupropion failed to block the discriminative effects of nicotine in all three studies (Wiley et al., 2002; Young and Glennon, 2002; Shoaib et al., 2003). These findings appear to correlate with the results of the various nicotine self-administration studies by indicating that bupropion does not ameliorate the acute subjective or reinforcing effects of nicotine. Whilst co-administration of mecamylamine blocked the stimulus effects of nicotine, it failed to antagonise the nicotine-like responses produced by bupropion (Wiley et al., 2002; Young and Glennon, 2002; Shoaib et al., 2003). This suggests that although bupropion shares similar discriminative stimulus effects to nicotine, their effects are not mediated by the same mechanisms of action. Bupropion probably produces nicotine-like responses through its ability to increase extracellular DA by inhibiting DAT.

1.7 Project aims

Section 1.6 highlights the uncertainties about the mechanism of action of bupropion in smoking cessation with observations from studies so far producing inconclusive results. Some suggest that its inhibition of DA and NA reuptake are fundamental to the efficacy of bupropion in the treatment of nicotine dependence, as it replaces the positive effects of nicotine during abstinence by maintaining raised extracellular levels of DA and NA in brain reward areas. Others propose that bupropion may aid smoking cessation through its ability to antagonise neuronal nAChRs, thereby attenuating the reinforcing properties of nicotine. These inconsistent findings have raised a few questions: (1) do these mechanisms of bupropion work in concert or does one predominate to help break the smoking habit, (2) are bupropion's mechanisms of action dependent on its concentration and/or type of model in which its effects are examined (*in vitro* vs. *in vivo*). With this in mind, the overall aim of this thesis is to systematically explore the interactions of nicotine and bupropion with respect to DA release, using various *in vitro* assays and *in vivo* microdialysis, together with locomotor activity, as models of increasing complexity.

The first part of the project (Chapter 2) examines the acute effects of bupropion on nAChR-evoked DA release *in vitro*. This is approached using two *in vitro* preparations of increasing complexity: synaptosomes that allow analysis of presynaptic terminals in isolation; and slices that maintain some anatomical integrity, thereby facilitating neurotransmitter crosstalk (see section 2.1.1.1). As a model system to study nAChR-evoked DA release, we utilised striatal preparations (see section 2.1.2) as they comprise DA terminals densely populated with nAChRs responsible for mediating the rewarding effects of nicotine. Therefore, the specific aims of this chapter are:

- To analyse the effects of bupropion on nicotine-evoked [^{3}H]DA release using an *in vitro* superfusion assay (see section 2.1.1).
- To probe the DAT and nAChR blocking properties of bupropion.
- To investigate how these effects of bupropion relate to the neuronal models in which they are examined.

The second part of the project (Chapter 3) elaborates on the findings from Chapter 2 by examining the acute effects of bupropion on nAChR-induced responses *in vivo*. This is approached by examining the interactions of bupropion and nicotine, with respect to DA-mediated responses, in more physiologically relevant models. Therefore, the specific aims of this chapter are:

- To examine whether the interactions of bupropion and nicotine observed *in vitro*, translate *in vivo*.
- To analyse the effects of bupropion on nicotine-evoked DA release in the NAc using *in vivo* microdialysis (see section 3.1.1), as DA release in the NAc is responsible for the motivational properties that maintain nicotine-seeking behaviour.
- To compare the neurochemical interactions of bupropion and nicotine to behavioural responses, by examining their effects on rat locomotor activity.

An additional part of the project was to construct an *in vivo* microdialysis set-up, and optimise a high pressure liquid chromatography (HPLC) system for the electrochemical detection (ECD) of DA, which is described in the Appendix.

CHAPTER 2

Actions of bupropion on nicotinic receptor-mediated [^{3}H]DA release

2.1 Introduction

The overall aim of this thesis was to study the interaction of bupropion with nicotine-evoked DA release. In this chapter, this is approached using *in vitro* methods that exploit the widespread occurrence of nAChRs at presynaptic locations (Wonnacott, 1997). Various experimental strategies adopted to monitor nAChR-evoked neurotransmitter release in brain preparations have shown presynaptic nAChRs to modulate the release of several neurotransmitters, including ACh, DA, NA, 5-HT, glutamate and GABA, from different brain regions (MacDermott et al., 1999). However, DA release from rodent striatum has been the most extensively studied example using neurochemical techniques, such as *in vitro* superfusion (Rapier et al., 1990; Soliakov et al., 1995; Prince et al., 1996; Kaiser et al., 1998; Wonnacott et al., 2000; Kulak et al., 2001; Grady et al., 2002).

2.1.1 *In vitro* superfusion

Superfusion allows the direct measurement of transmitter release from brain preparations (synaptosomes or slices) *in vitro*. The term "superfusion" was coined by J. H. Gaddum (1953), and refers to the flow of liquid, typically physiological buffer, over tissue, as opposed to perfusion in which liquid flows through the tissue (Gaddum, 1953). Usually several samples are superfused in parallel, and serial fractions of superfusate are collected for analysis of released transmitter. After establishing a stable baseline, transmitter release can be evoked by drug application or electrical stimulation in the presence or absence of antagonists or other drugs (see Fig 2.1 for the summary of a typical superfusion process). As most assay methods for endogenous transmitter are generally too insensitive to detect amounts in the individual fractions of superfusate, tissue is commonly loaded with radiolabelled transmitter prior to superfusion, and the release of radioactivity measured (Wonnacott et al., 2002). The validity of this approach rests largely on the assumption that radiolabelled transmitter accumulates in and is released from a transmitter pool that is identical to the one from which endogenous transmitter is released.

Automated superfusion systems, designed for brain slices, are produced commercially. Typically, customised systems that measure nicotine-evoked transmitter release over seconds to minutes are routinely used (Rapier et al., 1990; Prince et al., 1996; Wonnacott et al., 2000; Kulak et al., 2001; Grady et al., 2002). However, a superfusion system, with subsecond time resolution, has been developed (Turner et al., 1989) but there have been no published reports yet of nicotine-evoked transmitter release measured over such brief time scales (Wonnacott et al., 2002). Recently, a higher throughput release assay has been described (Puttfarcken et al., 2000). This is a static release system carried out in 96 well filter plates equipped with a support membrane to separate tissue slices from the bathing medium (containing the released transmitter) that can be removed by vacuum filtration (Wonnacott et al., 2002). This method has the added advantage of being useful for rapid screening of novel ligands, but unlike conventional superfusion systems, fails to provide a temporal profile for drug effects, and so is less suitable for the analysis of more complex interactions (e.g. effects of indirect agonists, such as bupropion, on transmitter release).

2.1.1.1 Synaptosomes or slices?

Synaptosomes were first identified by Hebb and Whittaker (1958) as sealed presynaptic nerve terminals, generated when brain tissue is homogenised under appropriate conditions (Whittaker, 1993). These structures retain all the features of the intact nerve ending making them the simplest system in which the complete chain of events from plasma depolarisation to transmitter release can be studied in isolation. Crude synaptosome preparations, produced by differential centrifugation, include free mitochondria and plasma membrane fragments, whilst highly purified preparations can be prepared by density gradient centrifugation that separates synaptosomes from membrane fragments and mitochondria (Wonnacott et al., 2002). As anatomical connections are severed in their preparation, superfusion of synaptosomes is considered to avoid cross-talk between nerve terminals, and so modulation of transmitter release reflects a direct effect via presynaptic receptors. However, preparations are rather frail, being prone to osmotic or mechanical damage. Typically, for investigating the effects of presynaptic nAChRs on transmitter release, a single stimulation with nicotinic agonist is given, and different agonist concentrations or the effect of antagonist or other drugs compared in parallel chambers (Wonnacott et al., 2002).

Slices or minces (about 250 µm) are conventionally prepared using an automated device, such as a McIlwain tissue chopper. They are quickly and easily prepared, and retain local anatomical integrity, making them more representative of the intact brain. The disadvantage of the latter aspect is that indirect effects on transmitter release, via synaptic connections, can complicate the interpretation of results. The protocol typically used to

study the effects of presynaptic nAChRs on transmitter release from slices is to determine and compare the absolute values of transmitter release (in cpm, typically expressed as a percentage of baseline or of total radiolabel originally accumulated by the preparation) between parallel chambers with or without various concentrations of drug of interest. Slice studies have been used for the pharmacological characterisation of nAChR-mediated [^{3}H]DA release in the striatum (Dwoskin et al., 1995; Marshall et al., 1996; Wonnacott et al., 2002).

2.1.2 Striatal preparations as a model system

The nicotinic modulation of [^{3}H]DA release from striatal preparations has been exploited as a model system for examining native nAChR responses (Wonnacott et al., 2000). Nicotinic agonists elicit Ca^{2+}-dependent [^{3}H]DA release from rat striatal synaptosomes in a concentration-dependent, mecamylamine-sensitive manner (Rapier et al., 1990; Grady et al., 1992; el Bizri and Clarke, 1994; Soliakov et al., 1995; Soliakov and Wonnacott, 1996), consistent with the activation of presynaptic nAChRs (Wonnacott, 1997). Insensitivity to the α7-selective antagonists, α-Bgt (Rapier et al., 1990; Grady et al., 1992) and α-conotoxin ImI (Kulak et al., 1997) argues against the direct involvement of α7 nAChR. Initial studies indicated that αCtxMII was a specific antagonist of nAChRs composed of α3 and β2 subunits (Cartier et al., 1996; Harvey et al., 1997). However, studies using transgenic mice, suggest that the toxin may recognise α6β2* nAChRs as well (see section 1.4.6.2; Champtiaux et al., 2002; Whiteaker et al., 2002). This toxin partially inhibited [^{3}H]DA release from rat striatal synaptosomes stimulated with nicotine (Kulak et al., 1997) or anatoxin-a (Kaiser et al., 1998), providing evidence for the heterogeneity of presynaptic nAChRs on DA terminals, with one population containing an α6β2 interface. Pharmacological studies with the novel partial agonist UB-165, led to the hypothesis that α4β2* nAChRs may comprise the other nAChR population on DA terminals (Sharples et al., 2000). This is consistent with the loss of [^{3}H]nicotine binding sites, following lesioning of the nigrostriatal pathway (Clarke and Pert, 1985). The requirement of the β2 subunit for the formation of the two putative subtypes of presynaptic nAChRs on striatal DA terminals is consistent with the localisation of β2 nAChR subunits immunoreactivity in most dopaminergic terminals in the dorsal striatum (Jones et al., 2001) and the absence of nicotine-evoked [^{3}H]DA release from synaptosomes or slices prepared from the striata of β2-null mutant mice (Grady et al., 2001; Zhou et al., 2001).

There is considerable *in vitro* evidence that glutamate can elicit DA release, by acting at AMPA and NMDA receptors on DA terminals in the striatum (Wang, 1991; Desce et al., 1992; Cheramy et al., 1996). Consistent with this view, is that locally applied nicotine can provoke striatal DA (Marshall et al., 1997) and glutamate release (Toth et al.,

1993), measured by *in vivo* microdialysis. Moreover, local application of NMDA antagonists diminish the ability of locally applied nicotine to elicit DA release *in vivo* (Toth et al., 1992). Recent studies on striatal slices, have interpreted an indirect nicotinic effect on [^{3}H]DA release, whereby stimulation of α7 nAChRs on glutamatergic terminals causes the release of glutamate that in turn promotes the release of DA via presynaptic glutamate receptors on dopaminergic terminals (Kaiser and Wonnacott, 2000). This component was not seen in synaptosomes, due to the absence of neurotransmitter crosstalk.

2.1.3 nAChRs and Ca^{2+} signalling

Intracellular Ca^{2+} has a pivotal role in regulating diverse aspects of neuronal processes such as transmitter release, excitability, synaptic plasticity and gene expression (Berridge, 1998; Dajas-Bailador and Wonnacott, 2004). Like other cells, neurones are endowed with a variety of voltage- and ligand-gated ion channels that normally serve as the principal Ca^{2+} entry pathway for cells. Activation of these channels allow Ca^{2+} entry down an electrochemical gradient resulting in elevated cytoplasmic free Ca^{2+} concentration above the normal range of 50-100 nM (Berridge, 1998). Additional Ca^{2+} entry routes across the plasma membrane involve the Na^+-Ca^{2+} exchange and store-operated Ca^{2+} channels. However, with regards to this thesis, the focus will be on voltage- and ligand-gated Ca^{2+}- channels.

There are a family of VOCC that are highly selective for Ca^{2+}. Plasma membrane depolarisation results in a rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and influx of Ca^{2+} through VOCC is the classical mechanism of transmitter release (Berridge, 1998). Characteristically, these channels also inactivate rapidly. A combination of electrophysiological and pharmacological criteria suggests that there are five distinct subtypes of VOCC: L, N, P, Q, R and T. The subtypes vary according to their activation and inactivation kinetics, conductance, and sensitivity to blocking agents. L stands for long-lasting due to the slow inactivation of the channel. L-channels are particularly important in regulating contraction of cardiac and smooth muscle. T stands for transient due to the fast inactivation of the channel. T-channels mediate Ca^{2+} entry into neurones, and thereby control various Ca^{2+}-dependent functions. N stands for neither long-lasting nor transient. N channels (and also P/Q) are involved in neurotransmitter and hormone release. R carries on alphabetically from N, P and Q, with O omitted. The location and function of R-channels is yet unknown (Rang et al., 2003).

In addition to VOCC, ligand-gated channels are the other major class of channels that facilitate Ca^{2+} entry. These are present in the plasma membrane of nerve terminals. The nAChR is a well-defined example of a ligand-gated channel with the capacity to elicit local changes in intracellular Ca^{2+}. The activation of this channel often results in a

substantial rise in intracellular Na^+, too. The relative permeability of nAChRs to Ca^{2+} over Na^+ is governed by the subunit composition, such that altering the molecular make-up of a channel has a substantial impact on the Ca^{2+} permeability (Rang et al., 2003). This is consistent with α7 nAChRs having the highest permeability to Ca^{2+} compared to other homomeric or heteromeric nAChRs (Seguela et al., 1993; Dajas-Bailador and Wonnacott, 2004).

Nicotine-evoked DA release requires the entry of extracellular Ca^{2+} into the presynaptic terminus, consistent with the activation of exocytotic mechanisms (Wonnacott, 1997). In principle, this could come about in two ways. Extracellular Ca^{2+} could enter directly through the ion pore of nAChRs by virtue of their intrinsic Ca^{2+} permeability. Alternatively, depolarisation of the presynaptic membrane produced by activation of nAChRs, would be sufficient to open VOCC associated with the release machinery, and Ca^{2+} entering through VOCC would be responsible for transmitter release. There is evidence for the operation of both mechanisms. In striatal DA synaptosomes bearing β2* nAChRs, nAChR-evoked DA release is mediated by VOCC (Prince et al., 1996; Soliakov and Wonnacott, 1996; Kulak et al., 2001). In contrast, activation of α3β4 nAChRs on hippocampal synaptosomes evokes the release of [^{3}H]noradrenaline without the involvement of VOCC (Kulak et al., 2001). Indeed, nAChRs also modulate transmitter release through other Ca^{2+}-dependent mechanisms that involve key signalling molecules such as protein kinases (Dajas-Bailador and Wonnacott, 2004). Also, in addition to the role of extracellular Ca^{2+}, there is increasing evidence that exocytosis may be regulated by Ca^{2+} release from intracellular stores (Dajas-Bailador et al., 2002).

2.1.4 SH-SY5Y cells as a model system

The human neuroblastoma SH-SY5Y cell line has been well characterised with respect to its complement of nAChRs. It expresses several nAChR subunits, including α3, α5, α7, β2 and β4, which form α3* and α7 subtypes of nAChRs (Lukas et al., 1993). Previous studies have shown that nAChR activation results in NA release through a cellular mechanism attributed to Ca^{2+} influx through the nAChR itself and VOCC (Wade et al., 1998). Additionally, it has also been shown that stimulation of SH-SY5Y cells with nicotinic agonists can increase $[Ca^{2+}]_i$ (Ridley et al., 2002). Together, these findings suggest that SH-SY5Y cells are a valuable neuronal model for investigating the cellular consequences of nAChR activation (Ridley et al., 2001; Dajas-Bailador et al., 2003; Barik et al., 2005).

Changes in $[Ca^{2+}]_i$ were first successfully measured by Ridgeway and colleagues in 1967. However, Tsien and colleagues in the 1980s produced a variety of chemical fluorescent indicators that provided reliable methods for measuring $[Ca^{2+}]_i$ (Takahashi et

al., 1999). The most widely used Ca^{2+} indicators are chemical fluorescent probes because their signal (light) is quite large for a given change in $[Ca^{2+}]_i$. For the study in this chapter, we utilised the non-ratiometric dye, Fluo-3, as its fluorescent properties were compatible with the instrument available. Fluo-3 is one of many Ca^{2+} indicators used to measure changes in fluorescence indicative of altered $[Ca^{2+}]_i$. Fluo-3 is essentially non-fluorescent unless bound to Ca^{2+}, after which it is reported to undergo a 40- to 200-fold increase in fluorescence (Takahashi et al., 1999). As fluo-3 is cell impermeable, it is typically derivatised with an AM ester that is cell permeable. Therefore, fluo-3 AM can passively diffuse across cell membranes, and once inside the cell, esterases cleave the AM group off the probe leaving a cell impermeable indicator. The AM esters have low aqueous solubility and so dispersing agents such as pluronic F127 are often used to facilitate cell loading. Pluronic F127 is a non-ionic dispersing agent that helps solubilise large dye molecules such as fluo-3 AM in physiological media (Takahashi et al., 1999).

2.1.5 Aims of this chapter

It is clear from various approaches that bupropion can interact with nAChRs (see section 1.6.2.3). What is uncertain, however, is how this interaction (that would decrease nicotine-evoked DA release) integrates with DAT blockade by bupropion (that would exert the opposite effect, namely an increase in extracellular DA). The present chapter aims to explore this relationship in two *in vitro* preparations of increasing complexity: striatal synaptosomes that allow analysis of DA terminals in isolation, and striatal slices that preserve some anatomical relationships that facilitate transmitter crosstalk.

Therefore, the particular aims of this chapter are:

- To explore the interactions of bupropion with nicotine-evoked DA release *in vitro.*
- First, using synaptosomes as the simplest system, to define nicotine-evoked [^{3}H]DA release and compare the effects of bupropion and nomifensine.
- Then, to extend the study to slices, allowing for more complex neurotransmitter crosstalk.
- To obtain a measure of the direct interaction of bupropion and nomifensine with nAChRs, by measuring Ca^{2+} fluorimetry in SH-SY5Y cells.

2.2 Materials and Methods

Cell Culture

Tissue culture media, serum and plastic ware were obtained from Gibco BRL (Paisley, UK). Media supplements were purchased from Sigma-Aldrich Co. Ltd (Gillingham, Dorset, UK). Fluo-3 AM and pluronic F127 were purchased from Molecular Probes (Eugene, Oregon, USA).

Drugs and Reagents

[7, 8- ^{3}H]dopamine ([^{3}H]DA; specificity 43-47 Ci/mmol, 1.67-1.81 TBq/mmol) was purchased from Amersham Biosciences Ltd (Bucks., UK). (-)-Nicotine hydrogen tartrate, bupropion, nomifensine, raclopride, mecamylamine, cadmium chloride and pargyline were purchased from Sigma-Aldrich Co. Ltd (Gillingham, Dorset, UK). DL-2-amino-5-phosphonopentanoic acid (AP-V) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were obtained from Tocris Cookson Ltd (Bristol, UK). α-Bgt was purchased from Molecular Probes (Cambridge, UK). All other chemicals were of analytical grade and obtained from standard commercial sources.

2.2.1 Superfusion of rat striatal synaptosomes and slices

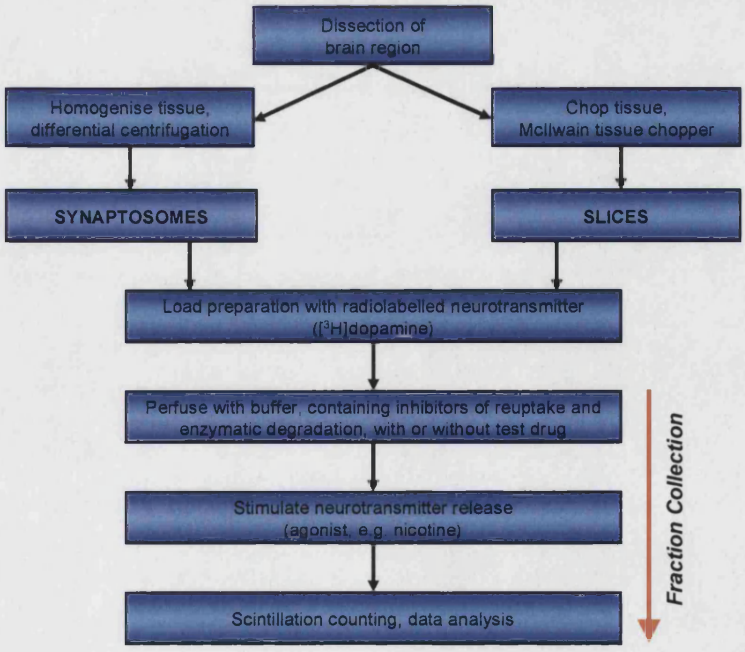


Fig. 2.1 Scheme summarising the steps involved in superfusion experiments with either synaptosomes or slices.

2.2.1.1 Dissection and preparation of striata

Male Sprague-Dawley rats (250-300 g) obtained from the University of Bath Animal House breeding colony, were killed by cervical dislocation, and the brains removed. Striata (80-120 mg wet tissue/rat) from two (for synaptosome preparations; Fig. 2.1) or three rats (for slice preparations; Fig. 2.1) were rapidly dissected and placed in ice-cold 0.32 M sucrose, pH 7.4. A crude P2 synaptosome preparation was prepared by differential centrifugation as previously described (Soliakov et al., 1995; Kaiser et al., 1998). In brief, tissue was homogenised in 0.32 M sucrose and centrifuged at 1000 x g for 10 min. The supernatant was decanted and centrifuged at 20,000 x g for 20 min to give a P2 pellet that was resuspended in Krebs buffer of the following composition: 118 mM NaCl, 2.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose, gassed with 95% O_2 / 5% CO_2, pH 7.4, supplemented with 1 mM ascorbic acid and 8 µM pargyline, to prevent DA degradation. The P2 synaptosomes were re-centrifuged at 20,000 x g for 10 min, resuspended in 1.5 ml Krebs buffer and incubated with [^{3}H]DA (0.1 µM, 0.185 MBq/ml) for 15 min at 37°C. Only for the preliminary nicotine-concentration response experiments, 0.5 µM nomifensine was added to the Krebs buffer, after the synaptosomes had been loaded with [^{3}H]DA, to inhibit DA re-uptake. The synaptosomes were then distributed, using a peristaltic pump, between 12 open superfusion chambers, each containing a double layer of Whatman GF/B filters, in a Brandel superfusion apparatus (Fig. 2.2).

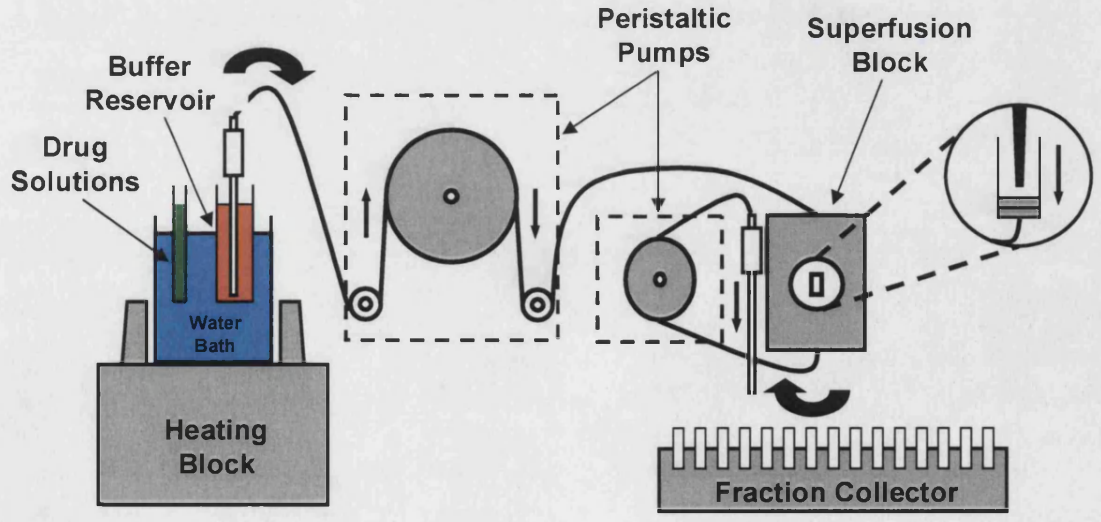


Fig. 2.2 Superfusion machine configured for the superfusion of synaptosomes. Arrows indicate the direction of buffer flow. The temperature of the heating block is adjusted such that the water bath is maintained at 37°C. ***Inset:*** Open superfusion chamber showing the double thickness of Whatman GF/B filters (grey) and the direction of buffer flow.

Striatal slices (0.25 mm prisms) were prepared as previously described (Kaiser and Wonnacott, 2000) using a McIlwain tissue chopper. The slices were rinsed in Krebs

buffer and incubated with [^{3}H]DA (0.1 µM, 0.185 MBq/ml) in 1.5 ml buffer for 15 min. The slices were diluted with Krebs buffer and excess radioactive label removed. Aliquots of the slice suspension (120 µl, 20-30 mg slices per chamber) were loaded into 12 closed chambers of a Brandel superfusion apparatus. A nylon mesh and GF/B filter were placed at each end of the chamber to prevent slices from flowing out of the apparatus (Fig. 2.3).

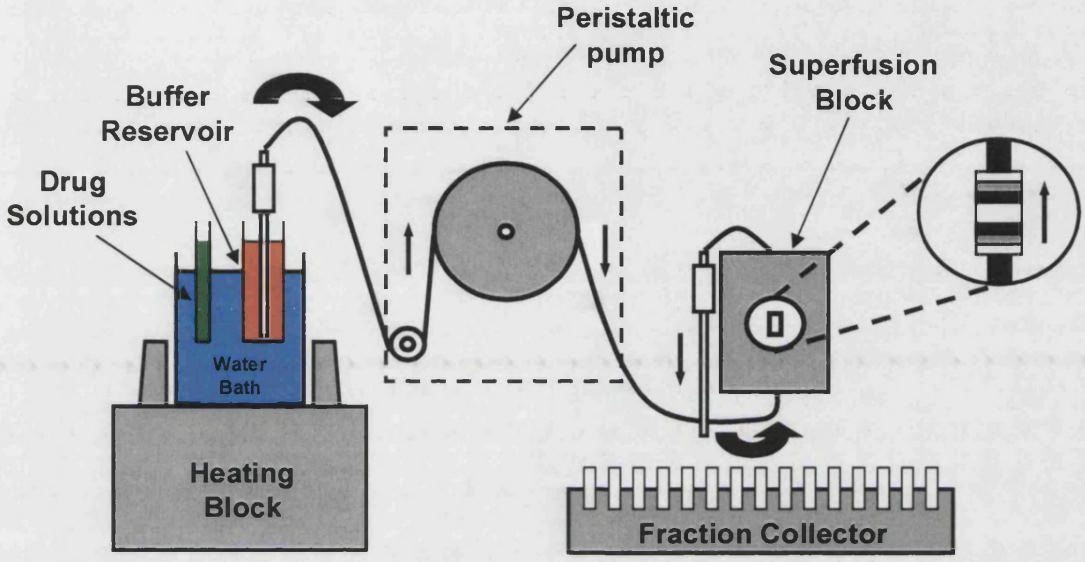


Fig. 2.3 Superfusion machine configured for the superfusion of slices.
Arrows indicate the direction of buffer flow. The temperature of the heating block is adjusted such that the water bath is maintained at 37°C. ***Inset:*** Closed superfusion chamber showing the position of Whatman GF/B filters (grey), nylon meshes (black) and the direction of buffer flow.

2.2.1.2 Drug application

Synaptosomes (open chambers) or slices (closed chambers) were superfused with Krebs buffer at a flow rate of 0.5 ml/min, at 37°C and serial fractions were collected every 2 min. After a 20 min washout period, synaptosomes or slices were superfused for a further 10 min in Krebs buffer with or without bupropion and / or test drug. In the case of α-Bgt (100 nM), the preincubation time was extended to 1 h. Nicotine was then applied for 40 s, separated from the bulk buffer flow by 10 s air bubbles, in the presence or absence of test drug. Superfusion was continued for a further 10 min in Krebs buffer with or without test drug.

2.2.1.3 Quantification of radioactivity

Radioactivity remaining in the synaptosomes or slices at the end of the experiment was determined by counting the GF/B filters from the superfusion chambers. In the case

of slices, solvable (200 µl) was added to the GF/B filters for 1 h, to lyse the slices, followed by addition of propan-2-ol (0.5 ml). Optiphase Safe™ scintillation fluid (4 ml) was added to all samples, and they were counted for radioactivity in a Packard TRI-CARD Liquid Scintillation Counter (model 1600; counting efficiency 44%).

2.2.1.4 Protein estimation

Protein content was determined using the Bio-Rad Protein Assay kit, which is essentially based on the method of Bradford (Bradford, 1976). The assay reagent was diluted 1:4 in distilled water and filtered through Whatman filter paper. A standard protein curve was constructed using samples (n = 7) of BSA, dissolved in Krebs buffer, over a concentration range of 0.2-1.4 mg/ml (Fig. 2.4). Brain samples were diluted 1:4 in Krebs buffer. Each sample (5 µl of BSA or diluted brain sample) was added to 8 wells in a 96 well plate and 250 µl of the assay reagent added to each well. Protein content was determined by measuring the optical density at 595 nm versus blank (Krebs buffer plus assay reagent) using an Anthos 2020 microplate reader (Anthos Labtec Instruments, Austria).

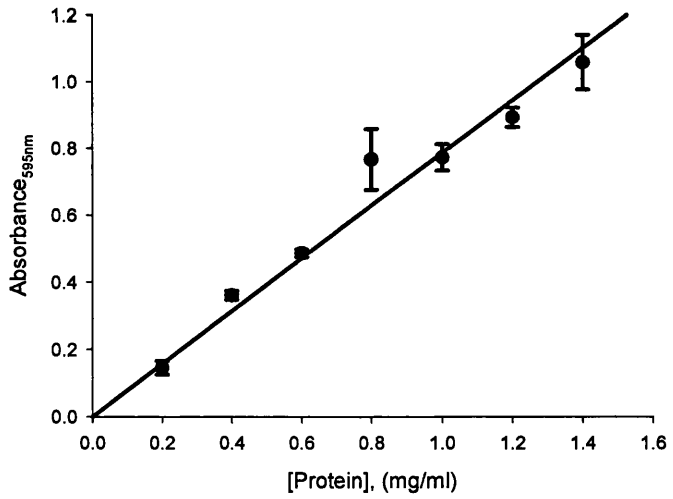


Fig. 2.4 Typical protein standard curve.
Points represent the mean absorbance ± S.E.M. obtained at 595 nm using increasing concentrations of BSA (0.2-1.4 mg/ml) assayed in a 96 well plate using the Bio-Rad protein assay.

2.2.2 Calcium fluorimetry

Human neuroblastoma SH-SY5Y cells (ECACC, Salisbury, UK; passages 14-20) were cultured as previously described (Dajas-Bailador et al., 2002). They were plated (1:5 dilution from culture media) into 96 well Primaria plates (Falcon, New Jersey, USA) and experiments performed 72 h afterwards with confluent cultures. After removal of the

medium from the confluent cultures, SH-SY5Y cells were washed twice with Tyrode's salt solution (TSS: 137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose; pH 7.4) and incubated with fluo-3 AM (10 μM) and 0.02% pluronic for 1 h at room temperature in the dark. Cells were then washed twice with TSS, before adding 80 μl buffer, with or without test drugs, per well. After 10 min pre-incubation at room temperature in the dark, changes in fluorescence (excitation 485 nm, emission 538 nm) were measured using a Fluroskan Ascent fluorescent plate reader (Labsystems, Helsinki, Finland).

Basal levels of fluorescence were monitored for 5 s before addition of an agonist (20 μl). Changes in fluorescence were monitored for a further 20 s. For normalising fluorescence signals, responses from each well were calibrated by determination of the maximum and minimum fluorescence values at the end of each experiment, by addition of 0.2% Triton X100 (F_{max}), followed by 40 mM $MnCl_2$ (F_{min}). Data were calculated as a percentage of $F_{max} - F_{min}$. Values in the presence of test drugs were expressed as a percentage of the control response to stimulus (nicotine or KCl; included in all experiments).

2.2.3 Data analysis

2.2.3.1 Superfusion

The baseline was derived by fitting the following double exponential decay equation to the data, using SigmaPlot (v 2.0) for Windows:

$$y = ae^{-bx} + ce^{-dx}$$

where *a* and *c* are the initial (x = 0) release in each phase, *b* and *d* are the decay constants in each phase, and *x* is the fraction number (Soliakov et al., 1995). For comparison between different experiments, superfusion profiles were normalised as percentages of fitted baselines.

Evoked [^{3}H]DA release was calculated as the area under the peak, after subtraction of the baseline, and expressed as a percentage of the total radioactivity present in the synaptosomes or slices at the time of agonist stimulation (the sum of subsequently released [^{3}H]DA plus that remaining on the filters at the end of the experiment) to give fractional release. Data are also presented by expressing fractional release as a percentage of the corresponding control, assayed in parallel. Values represent the mean ± S.E.M. of the number of independent experiments carried out; each experiment consisted of two or three replicate chambers for each condition.

To examine concentration-inhibition relationships, data were fitted to the Hill Equation (see below) using the non-linear least squares curve fitting function of SigmaPlot:

$$\text{Percentage release} = \frac{100\%}{1+\left(\frac{[Drug]}{IC_{50}}\right)^{n_H}}$$

where n_H is the Hill Number, [*Drug*] is the concentration of the antagonist, and IC_{50} is the concentration at which half-maximal inhibition of [^{3}H]DA release by antagonist was obtained. To examine concentration-response relationships, data were fitted to the above Hill Equation, and IC_{50} values were replaced by EC_{50} values. EC_{50} is the concentration at which half-maximal [^{3}H]DA release in response to agonist was obtained.

Statistical analyses were performed using the unpaired Student's t-test or one-way ANOVA with Dunnett's post-hoc test, as stated in the figure legends. Values of $p<0.05$ were taken to be statistically significant.

2.2.3.2 Calcium fluorimetry

To examine concentration-inhibition relationships, data were fitted to the Hill Equation (as in the superfusion experiments - see section 2.2.3.1) using the non-linear least squares curve fitting function of SigmaPlot, where n_H is the Hill Number, [*Drug*] is the concentration of the antagonist, and IC_{50} is the concentration at which half-maximal change in fluorescence by antagonist was obtained.

Values represent the mean ± S.E.M. of three or more independent experiments (each with four replicates). Statistical analyses were performed using the unpaired Student's t-test or one-way ANOVA with Dunnett's post-hoc test, as stated in the figure legends. Values of $p<0.05$ were taken to be statistically significant.

2.3 Results

2.3.1 Superfusion of striatal synaptosomes and slices

2.3.1.1 Nicotine concentration-response relationship

To monitor the reproducibility of the *in vitro* superfusion assay in comparison with previous results from the laboratory (Kaiser et al., 1998; Wonnacott et al., 2000), a preliminary experiment examining nicotine-evoked [^{3}H]DA release from rat striatal synaptosomes was conducted (Fig. 2.5). A 40 s pulse of nicotine (10 µM) elicited a peak of radioactivity above basal release that was almost totally abolished in the presence of the general nAChR antagonist, mecamylamine (10 µM; Fig. 2.5a). Firstly a range of concentrations of nicotine (0.03-100 µM) was tested to yield a dose-response curve for nicotine-evoked [^{3}H]DA release (Fig. 2.5b). An EC_{50} value of 4.8 ± 0.1 µM nicotine was obtained. Next, a similar range of concentrations of nicotine (0.3-100 µM) was tested in the presence of 10 µM mecamylamine to distinguish nAChR-mediated and non-specific [^{3}H]DA release (Fig. 2.5c). These data were expressed as a percentage of the response to a 10 µM nicotine control, since this concentration elicited responses that were just below the maximum on the dose-response curve (Fig. 2.5b). Fig. 2.5c shows that mecamylamine (10 µM) blocked the response to a range of concentrations of nicotine-evoked [^{3}H]DA release (0.3-100 µM), but failed to do so at the highest concentration of nicotine (100 µM).

2.3.1.2 Effect of DA reuptake inhibitors on nicotine-evoked [^{3}H]DA release

[^{3}H]DA release profiles

To explore the interaction between bupropion and nicotine with respect to [^{3}H]DA release, the effect of bupropion (0.1-10 µM) on nicotine-evoked [^{3}H]DA release was examined in rat striatal synaptosomes and slices, by superfusion. To study bupropion's ability to block DA reuptake, it was compared with a well-defined DAT inhibitor, nomifensine (10 µM), thereby representing a positive control. [^{3}H]DA release was evoked by 10 µM nicotine (as this concentration was sensitive enough to detect any drug-induced changes above or below this level; Fig. 2.5b) in the absence (experimental control) and presence of test drug. Initially, nomifensine (10 µM) was examined for its effect on nicotine-evoked [^{3}H]DA release, in synaptosomes and slices, by superfusion. The effects

of both nomifensine and bupropion are illustrated firstly as typical superfusion profiles (Figs 2.6 and 2.7), and then interpreted quantitatively, by histograms (Fig. 2.8).

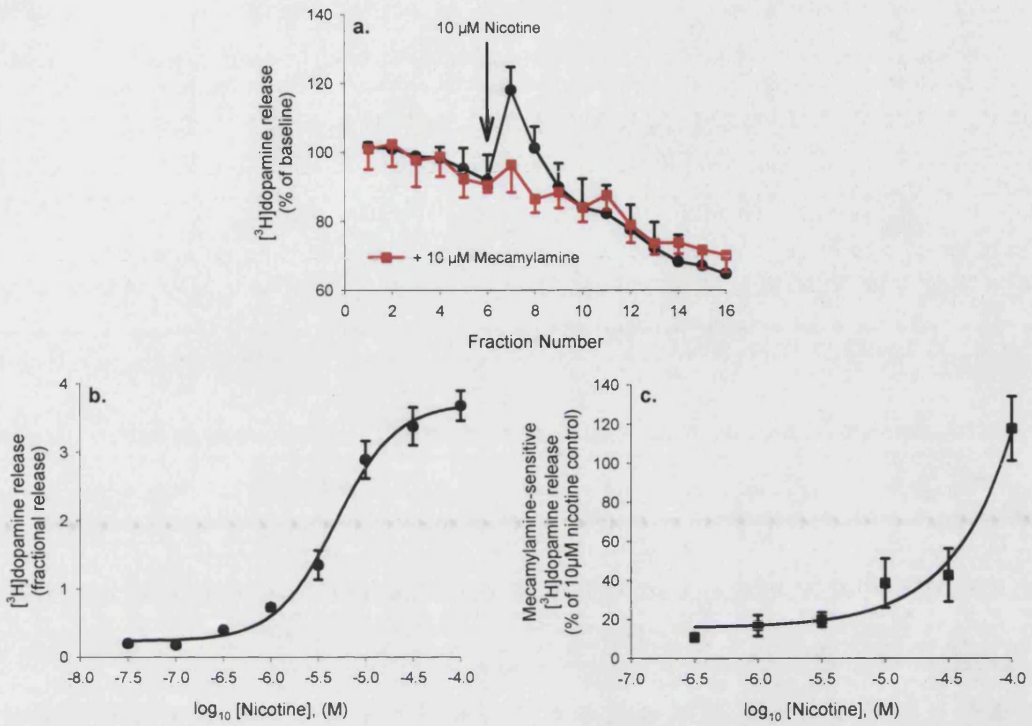


Fig. 2.5 Nicotine-evoked [^{3}H]DA release from striatal synaptosomes.
Synaptosomes were loaded with [^{3}H]DA and superfused as described in section 2.2.1.2. Mecamylamine was added to the perfusing buffer 10 min prior to stimulation with nicotine for 40 s; 2 min (1 ml) serial fractions were collected and counted for radioactivity. Nicotine-evoked responses in the absence of mecamylamine were determined in parallel chambers. Representative superfusion profiles show evoked [^{3}H]DA release from striatal synaptosomes in the absence (•) and presence (■) of 10 µM mecamylamine (a). Mean values from a single experiment are expressed as a percentage (± S.E.M. of triplicate chambers) of the mean basal release. Concentration-response curves for [^{3}H]DA release in response to nicotine in the absence (b) and presence (c) of 10 µM mecamylamine. Evoked [^{3}H]DA release was calculated as a fraction of total radioactivity present at the time of stimulation and presented as raw fractional release (b) or percentage of the response to 10 µM nicotine alone (c), measured in parallel. All values are the mean ± S.E.M. of at least three separate experiments, each consisting of two or more replicate chambers. Concentration-response data were fitted to the Hill Equation, as described in section 2.2.3.1. The EC_{50} value for nicotine-evoked [^{3}H]DA release (b) was 4.8 ± 0.1 µM.

A 40 s pulse of 10 µM nicotine elicited a peak of radioactivity above basal release that was affected, in the presence of 10 µM nomifensine. The effect of nomifensine was markedly different in synaptosomes (Fig. 2.6a) as compared to slices (Fig. 2.6c). In synaptosomes, nicotine-evoked [^{3}H]DA release in the presence of nomifensine, exhibited a similar peak height but a narrower peak shape with respect to control. In slices, however, evoked release in the presence of nomifensine exhibited a similar peak shape but an increased peak height, as well as an increased baseline, with respect to control (The interpretations of these results are considered later in this section).

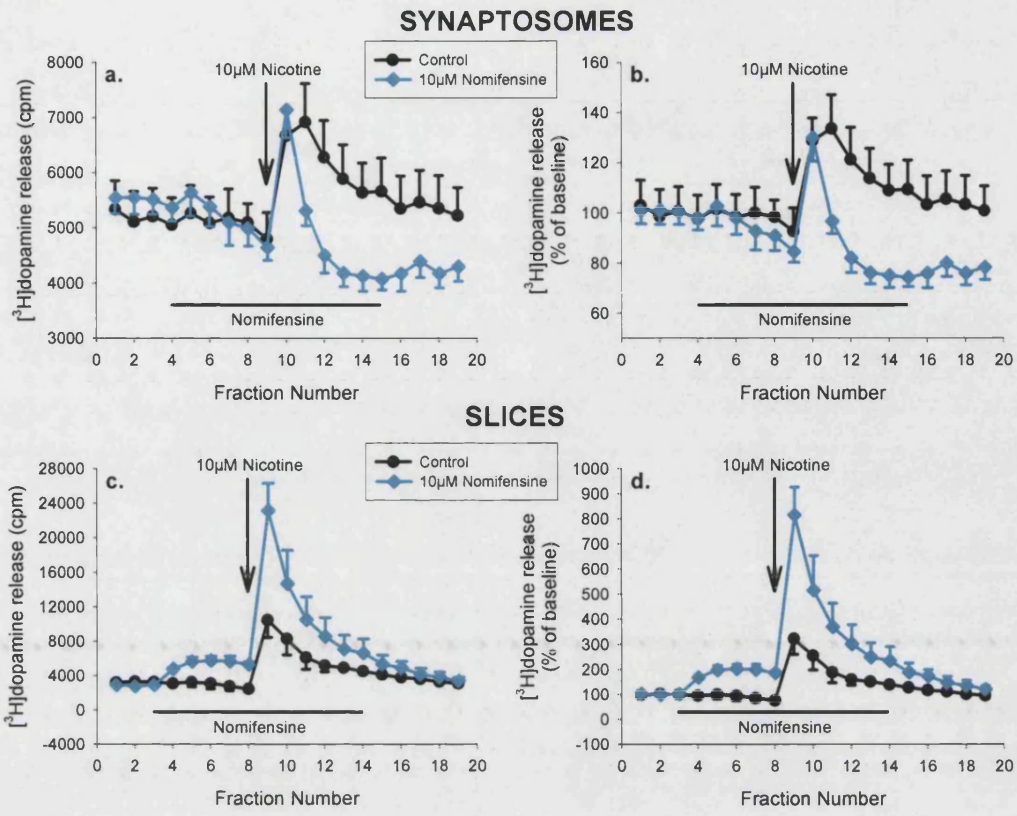


Fig. 2.6 Superfusion profiles showing the effect of nomifensine on nicotine-evoked [^{3}H]DA release from striatal synaptosomes and slices.

Synaptosomes (a, b) and slices (c, d) were loaded with [^{3}H]DA and superfused as described in section 2.2.1.2. Nomifensine (10 μM) was added to the perfusing buffer 10 min prior to stimulation with 10 μM nicotine for 40 s; 2 min (1 ml) serial fractions were collected and counted for radioactivity. Nicotine-evoked responses in the absence of nomifensine were determined in parallel chambers. Mean values (± S.E.M. of triplicate chambers) from a single experiment are expressed as raw cpm (a, c) or percentage of the mean basal release (b, d). All values are the mean ± S.E.M. of at least three separate experiments, each consisting of two or more replicate chambers.

The effects of bupropion (0.1, 10 μM) on nicotine-evoked [^{3}H]DA release in synaptosomes (Fig. 2.7a) were also different to those in slices (Fig. 2.7c). In synaptosomes, the effect of 0.1 μM bupropion was difficult to differentiate from that of control, whilst 10 μM bupropion produced a lower peak height with respect to control. Both concentrations of bupropion produced variations in baseline when compared to that of control. In slices, both concentrations of bupropion (0.1, 10 μM) produced lower peak heights and similar peak shapes, with respect to control (The interpretations of these results are considered later in this section).

To clarify the different effects of nomifensine and bupropion, evoked [^{3}H]DA release was normalised as a percentage of a fitted baseline (Fig. 2.6b, d for nomifensine, Fig. 2.7b, d for bupropion), where the mean basal release prior to superfusion with test drug, was expressed as 100%. This is because at this point, all drug treatments, in the presence or absence of test drug, were subjected to the same experimental condition, i.e. superfusion in Krebs buffer only (as described in section 2.2.1.2). Note that the y-axes of

Fig. 2.6b vs. 2.6d and Fig. 2.7b vs. 2.7d differ, reflecting the greater efficacy of drugs in the slice preparation.

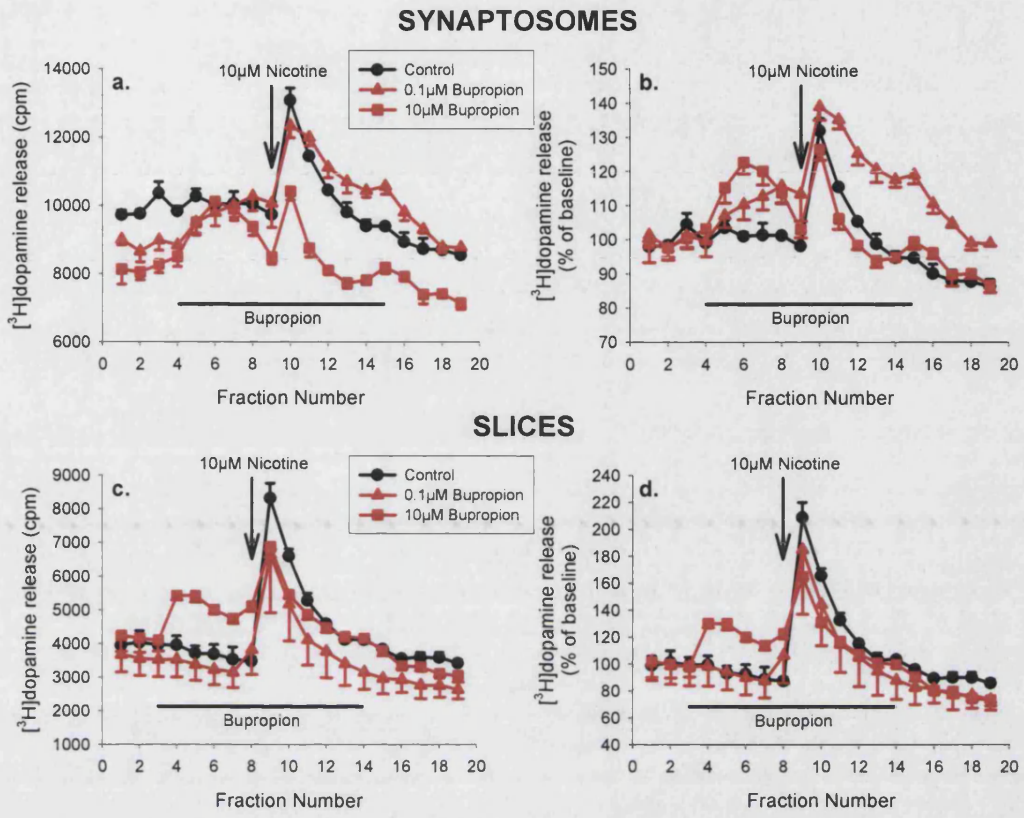


Fig. 2.7 Superfusion profiles showing the effect of bupropion on nicotine-evoked [^{3}H]DA release from striatal synaptosomes and slices.

Synaptosomes (a, b) and slices (c, d) were loaded with [^{3}H]DA and superfused as described in section 2.2.1.2. Bupropion (0.1 μM or 10 μM) was added to the perfusing buffer 10 min prior to stimulation with 10 μM nicotine for 40 s; 2 min (1 ml) serial fractions were collected and counted for radioactivity. Nicotine-evoked responses in the absence of bupropion were determined in parallel chambers. Mean values (± S.E.M. of triplicate chambers) from a single experiment are expressed as raw cpm (a, c) or percentage of the mean basal release (b, d). All values are the mean ± S.E.M. of at least three separate experiments, each consisting of two or more replicate chambers.

In synaptosomes, both concentrations of bupropion increased basal release (Fig. 2.7b), with a slowly increasing effect in response to 0.1 μM bupropion and a rapid but transient increase in basal levels of [^{3}H]DA in response to 10 μM bupropion. In slices, a rapid increase in basal levels of [^{3}H]DA was seen in response to 10 μM nomifensine (Fig. 2.6d) and 10 μM bupropion (Fig. 2.7d).

Effect of nomifensine on nicotine-evoked [^{3}H]DA release

In synaptosomes, nomifensine (10 μM) significantly inhibited nicotine-evoked [^{3}H]DA release by 36.8 ± 5.7% ($p<0.05$, $n = 9$; Fig. 2.8a). Note that in Fig. 2.6b, nicotine-evoked [^{3}H]DA release in the absence and presence of 10 μM nomifensine, showed similar peak heights, but in the presence of 10 μM nomifensine, exhibited a narrower peak

shape with respect to control. Evoked release was calculated as the total radioactivity present under the peak, and expressed as a percentage of the mean basal release, and thus attributed to the significant decrease seen in nicotine-evoked [^{3}H]DA release in the presence of 10 µM nomifensine (Fig. 2.8a).

In slices, nomifensine had the opposite effect to that seen in synaptosomes. It significantly increased the extracellular amount of [^{3}H]DA evoked by 10 µM nicotine, by 84.4 ± 22.9% ($p<0.05$, n = 13; Fig. 2.8b). This was attributed to the increased peak height in the presence of nomifensine, with respect to control (Fig. 2.6d).

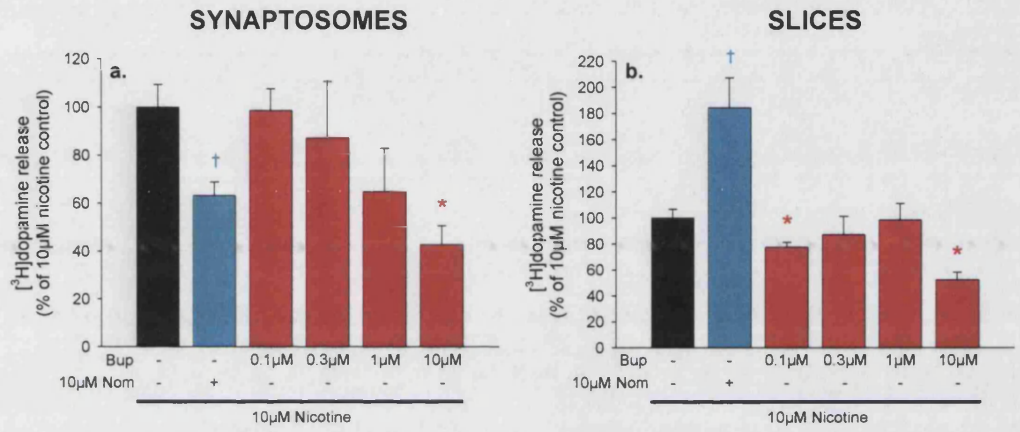


Fig. 2.8 Effect of DA reuptake inhibitors on nicotine-evoked [^{3}H]DA release from striatal synaptosomes (a) and slices (b).

Synaptosomes and slices were loaded with [^{3}H]DA and superfused as described in section 2.2.1.2. Bupropion (Bup; 0.1-10 µM) or nomifensine (Nom; 10 µM) were added to the perfusing buffer 10 min prior to stimulation with 10 µM nicotine for 40 s; 2 min (1 ml) serial fractions were collected and counted for radioactivity. Data were calculated as fractional release and are expressed as a percentage of the response to 10 µM nicotine alone (control), determined in parallel. Values are the mean ± S.E.M. of at least three separate experiments, each consisting of two or more replicate chambers ($^{\dagger}p<0.05$, $^{*}p<0.05$ significantly different from control response; unpaired Student's t-test, and one-way ANOVA with Dunnett's post-hoc test, respectively).

Effect of bupropion on nicotine-evoked [^{3}H]DA release

In synaptosomes, bupropion exerted a concentration-dependent effect on nicotine-evoked [^{3}H]DA release (Fig. 2.8a), with the low concentration of 0.1 µM showing no significant difference in [^{3}H]DA levels with respect to control; and the high concentration of 10 µM causing a significant inhibition by 57.3 ± 7.9% ($p<0.05$, n = 6). The presence of 10 µM bupropion produced a lower peak height with respect to control (Fig. 2.7b), and so accounted for the significant decrease in evoked [^{3}H]DA release (Fig. 2.8a).

In slices, nicotine-evoked [^{3}H]DA release was reduced in the presence of both concentrations of bupropion (0.1, 10 µM; Fig. 2.7d). The concentration response relationship for the effect of bupropion on nicotine-evoked [^{3}H]DA release has an inverted u-shape (Fig. 2.8b): in contrast to the results from synaptosomes, nicotine-evoked [^{3}H]DA release was significantly decreased in the presence of 0.1 µM bupropion, by 22.5 ± 3.8%

($p<0.05$, n = 6). At the highest concentration of bupropion (10 µM) there was a significant inhibition of nicotine-evoked [^{3}H]DA release by 47.6 ± 5.7% ($p<0.05$, n = 6), comparable to that observed with synaptosomes. The intermediate concentrations of bupropion had no significant influence on nicotine-evoked [^{3}H]DA release. Both 0.1 µM and 10 µM bupropion exhibited similar peak shapes as compared to control, and showed lower peak heights (Fig. 2.7d), consistent with the decrease in nicotine-evoked [^{3}H]DA release (Fig. 2.8b).

Combined effect of bupropion and nomifensine on nicotine-evoked [^{3}H]DA release

Due to the contrasting effects of nomifensine on nicotine-evoked [^{3}H]DA release in synaptosomes and slices (Fig. 2.8), the effect of increasing concentrations of bupropion (0.1-10 µM) in combination with 10 µM nomifensine on nicotine-evoked [^{3}H]DA release was examined in rat striatal synaptosomes and slices, by superfusion (Fig. 2.9). Each experiment was performed in the presence of 10 µM nomifensine alone as a positive control, as well as in the absence of any DAT inhibitor (10 µM nicotine control).

The dual effect of 10 µM nomifensine and bupropion significantly decreased nicotine-evoked [^{3}H]DA release in synaptosomes by 38.4 ± 6.5%, 33.6 ± 4.2%, 46.4 ± 15.9% and 75.5 ± 2.5% ($p<0.05$, n = 3) at 0.1 µM, 0.3 µM, 1 µM and 10 µM bupropion, respectively (Fig. 2.9a). Nomifensine alone, also exhibited a similar level of inhibition, to that observed when it was present with either 0.1µM, 0.3µM or 1µM bupropion. Approximately two-fold greater inhibition was seen when co-perfused with 10 µM bupropion. Thus, to clarify the effect of bupropion in this combination treatment, results were expressed as a percentage of the response to 10 µM nicotine in the presence of 10 µM nomifensine alone (Fig. 2.9b), on nicotine-evoked [^{3}H]DA release. Here, the effect of bupropion was concentration-dependent, exhibiting a significant decrease in evoked release at the highest concentration (10 µM) by 41.8 ± 14.9% ($p<0.05$, n = 4); a pattern that was similar to that seen in Fig. 2.8a, when increasing concentrations of bupropion alone were examined for their effect on nicotine-evoked [^{3}H]DA release.

In slices, the dual effect of 10 µM nomifensine and bupropion exhibited a concentration-dependent profile with significant increases in evoked [^{3}H]DA release by 122.5 ± 84.0%, 92.3 ± 43.2% and 75.3 ± 8.2% ($p<0.05$, n = 3) at 0.1 µM, 0.3 µM and 1 µM bupropion, respectively (Fig. 2.9c). When the results were expressed as a percentage of the response to 10 µM nicotine in the presence of 10 µM nomifensine alone (Fig. 2.9d), on nicotine-evoked [^{3}H]DA release, the effect of bupropion was concentration-dependent, exhibiting a significant decrease in evoked release at 10 µM bupropion by 53.0 ± 8.7% ($p<0.05$, n = 3), respectively. This concentration-dependent effect of bupropion was however, in contrast to the inverted u-shaped profile seen in Fig. 2.8b, when increasing concentrations of bupropion alone, were examined for their effect on nicotine-evoked [^{3}H]DA release.

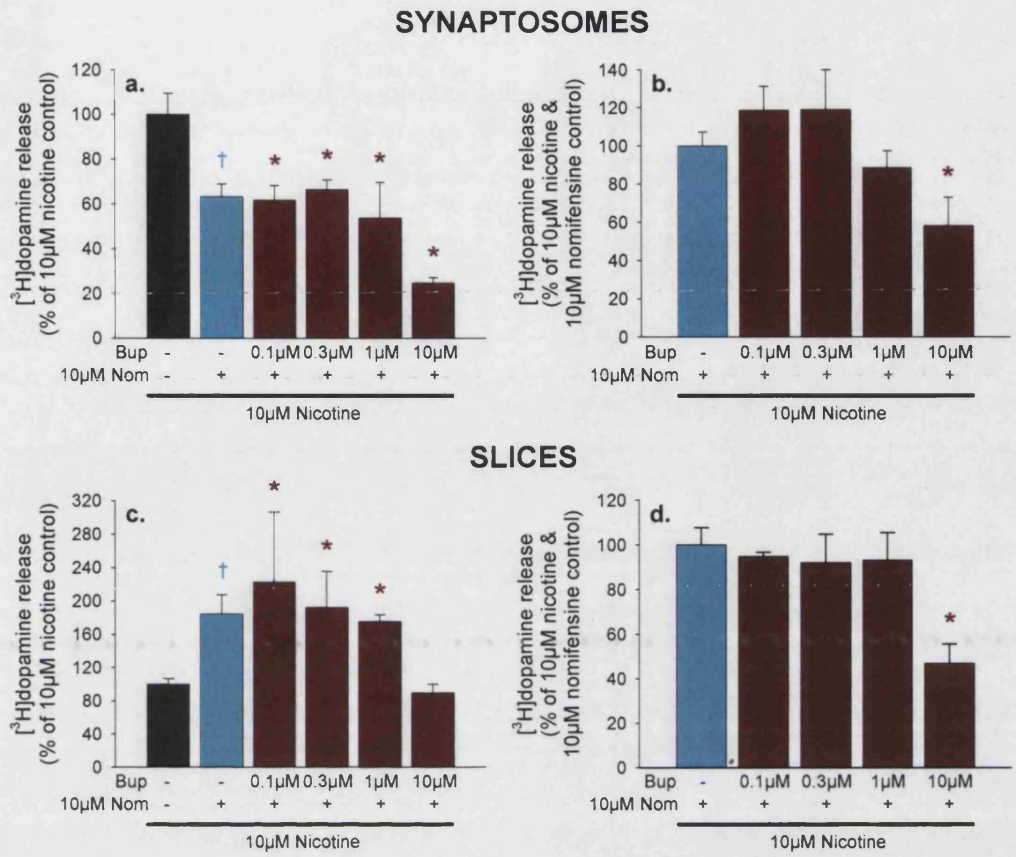


Fig. 2.9 Combined effect of bupropion and nomifensine on nicotine-evoked [^{3}H]DA release from striatal synaptosomes (a, b) and slices (c, d).
Synaptosomes and slices were loaded with [^{3}H]DA and superfused as described in section 2.2.1.2. Nomifensine (Nom; 10 μM) alone, or nomifensine (10 μM) in combination with bupropion (Bup; 0.1-10 μM) were added to the perfusing buffer 10 min prior to stimulation with 10 μM nicotine for 40 s; 2 min (1 ml) serial fractions were collected and counted for radioactivity. Data were calculated as fractional release and are expressed as a percentage of the response to 10 μM nicotine alone (control; a, c) or 10 μM nicotine in the presence of 10 μM nomifensine (control; b, d), determined in parallel. Values are the mean ± S.E.M. of at least three separate experiments, each consisting of two or more replicate chambers ($^{\dagger}p<0.05$, $^{*}p<0.05$ significantly different from control response; unpaired Student's t-test, and one-way ANOVA with Dunnett's post-hoc test, respectively).

2.3.1.3 DA D2 receptor-induced modulation of bupropion-mediated effects on nicotine-evoked [^{3}H]DA release

To determine if inhibitory DA D2 receptors contributed to the concentration-dependent effects of bupropion on nicotine-evoked [^{3}H]DA release in striatal synaptosomes (Fig. 2.8a) and slices (Fig. 2.8b), raclopride (10 nM) was co-applied with 0.1 μM and 10 μM bupropion (Fig. 2.10). This concentration of raclopride was selected as it was sufficient to induce a complete blockade of DA D2 receptors (K_d = 1.8 nM; Professor Philip Strange – personal communication). Raclopride alone did not significantly affect nicotine-evoked [^{3}H]DA release in synaptosomes or slices. Moreover, in

synaptosomes in the presence of raclopride, 10 µM bupropion inhibited nicotine-evoked [^{3}H]DA release by the same extent as in the absence of raclopride (Fig. 2.10a). In slices, however, raclopride reversed the decrease in nicotine-evoked [^{3}H]DA release observed in the presence of 0.1 µM bupropion ($p<0.05$, n = 3), while having no significant effect on the diminished response in the presence of 10 µM bupropion (Fig. 2.10b).

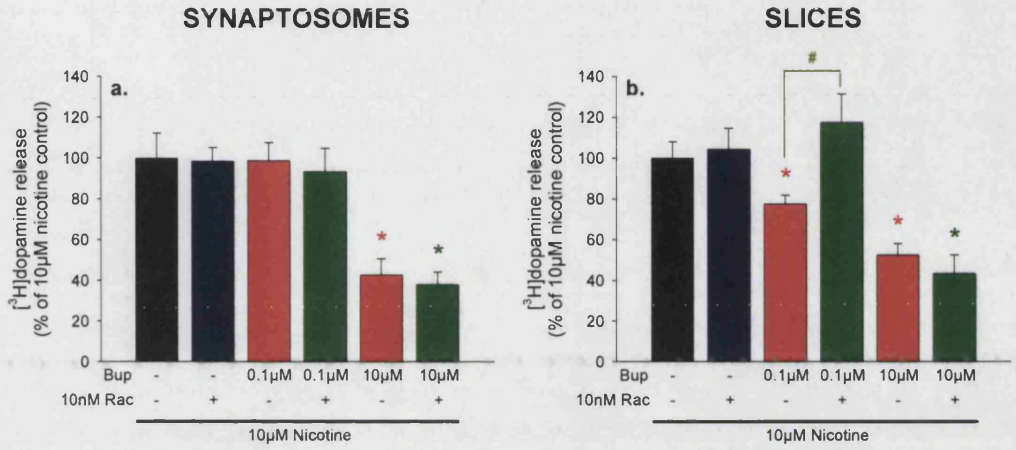


Fig. 2.10 Contribution of D2 receptors on the influence of bupropion on nicotine-evoked [^{3}H]DA release from striatal synaptosomes (a) and slices (b).
Synaptosomes and slices were loaded with [^{3}H]DA and superfused as described in section 2.2.1.2. Raclopride (Rac; 10 nM) alone, bupropion (Bup; 0.1 µM or 10 µM) alone, or raclopride (10 nM) in combination with bupropion (0.1 µM or 10 µM) were added to the perfusing buffer 10 min prior to stimulation with 10 µM nicotine for 40 s; 2 min (1 ml) serial fractions were collected and counted for radioactivity. Data were calculated as fractional release and are expressed as a percentage of the response to 10 µM nicotine alone (control), determined in parallel. Values are the mean ± S.E.M. of at least three separate experiments, each consisting of two or more replicate chambers ($^*p<0.05$ significantly different from control response, one-way ANOVA with Dunnett's post-hoc test; $^\#p<0.05$, significantly different from response in the presence of 0.1 µM bupropion alone, unpaired Student's t-test).

2.3.1.4 Role of ionotropic glutamate receptors and α7 nAChRs on bupropion-induced decrease in nicotine-evoked [^{3}H]DA release from striatal slices

Previous studies have provided evidence for the indirect modulation of [^{3}H]DA release from rat striatal slices through the nicotine-evoked release of glutamate (Kaiser and Wonnacott, 2000). To determine if this component is modified in the presence of bupropion, two ionotropic glutamate receptor antagonists were compared for their abilities to influence the decrease in nicotine-evoked [^{3}H]DA release from striatal slices observed in the presence of the lower concentration of bupropion (0.1 µM; Fig. 2.11a).

On its own, the AMPA/kainate receptor antagonist DNQX (100 µM) significantly decreased nicotine-evoked [^{3}H]DA release by 32.2 ± 11.2% ($p<0.05$, n = 5, Fig. 2.11a),

consistent with a proportion of nicotine-evoked [^{3}H]DA release mediated by the release of glutamate from neighbouring terminals. When DNQX and bupropion (0.1 µM) were co-applied, nicotine-evoked [^{3}H]DA release was reduced by 14.5 ± 9.3%, but this was not significantly different from control. In contrast, the NMDA receptor antagonist AP-V (100 µM), did not significantly affect nicotine-evoked [^{3}H]DA release when applied on its own. As the Krebs buffer contained Mg^{2+} but lacked glycine, NMDA receptors may not have been activated (Cheramy et al., 1996). However, in the presence of 0.1 µM bupropion and AP-V (100 µM), nicotine-evoked [^{3}H]DA release was decreased by 49.2 ± 9.6% ($p<0.05$, n = 4; Fig. 2.11a). This was not significantly different from the decrease observed in the presence of bupropion alone.

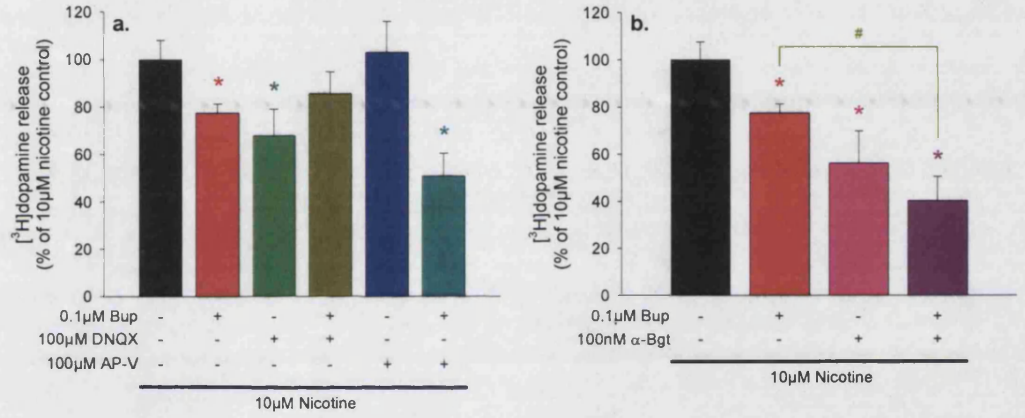


Fig. 2.11 Contribution of ionotropic glutamate receptors (a) and α7 nAChRs (b) in the presence of 0.1 µM bupropion to nicotine-evoked [^{3}H]DA release from striatal slices. Slices were loaded with [^{3}H]DA and superfused as described in section 2.2.1.2. Bupropion (Bup; 0.1 µM) alone, DNQX (100 µM) alone, AP-V (100 µM) alone, or bupropion (0.1 µM) in combination with either DNQX (100 µM) or AP-V (100 µM) were added to the perfusing buffer 10 min prior to stimulation with 10 µM nicotine for 40 s. For α-bungarotoxin (α-Bgt; 100 nM), the preincubation time was extended to 1 h. Two min (1 ml) serial fractions were collected and counted for radioactivity. Data were calculated as fractional release and are expressed as a percentage of the response to 10 µM nicotine alone (control), determined in parallel. Values are the mean ± S.E.M. of at least three separate experiments, each consisting of two or more replicate chambers ($^*p<0.05$ significantly different from control response, one-way ANOVA with Dunnett's post-hoc test; $^\#p<0.05$, significantly different from response in the presence of 0.1 µM bupropion alone, unpaired Student's t-test).

The indirect modulation of nicotine-evoked [^{3}H]DA release by glutamate is controlled by α7 nAChRs postulated to reside on striatal glutamatergic nerve terminals (Kaiser and Wonnacott, 2000). In agreement with this hypothesis, the α7-selective nAChR antagonist α-Bgt (100 nM) significantly blocked nicotine-evoked [^{3}H]DA release by 43.7 ± 13.6% ($p<0.05$, n = 4; Fig. 2.11b). Co-application of α-Bgt (100 nM) with bupropion (0.1 µM) resulted in a decrease in nicotine-evoked [^{3}H]DA release of 59.7 ± 10.3% ($p<0.05$, n = 4; Fig. 2.11b). This was a significantly greater decrease than observed in the presence of bupropion alone.

2.3.2 Calcium fluorimetry

To examine the initial response to nAChR activation, drug-induced changes in fluorescence as an index of $[Ca^{2+}]_i$ were measured in SH-SY5Y cells using fluorimetry (Dajas-Bailador et al., 2002). Evoked responses in the presence of nomifensine alone, bupropion alone, and the combination of nomifensine and bupropion, were examined in this cell line. Receptor and VOCC activation was induced by stimulation with nicotine in the absence and presence of the above drugs; whilst any effect on VOCC only, was examined by stimulation with KCl in the absence and presence of the above drugs.

2.3.2.1 Effect of DA reuptake inhibitors on Ca^{2+} fluxes in SH-SY5Y cells

Nomifensine-induced effects on Ca^{2+} fluxes in SH-SY5Y cells

Due to the unexpected inhibition by 10 µM nomifensine on nicotine-evoked [^{3}H]DA release in rat striatal synaptosomes (Fig. 2.8a), pre-incubation with nomifensine was examined for its effects on nicotine- or KCl-evoked changes in fluorescence in SH-SY5Y cells (Fig. 2.12). Nomifensine (0.5-10 µM) concentration-dependently inhibited increases in fluorescence evoked by 30 µM nicotine, by 19.8 ± 7.9% and 31.3 ± 4.4% ($p<0.05$, $n = 5$ and $n = 9$) at 1 µM and 10 µM, respectively (Fig. 2.12a). Furthermore, 10 µM nomifensine significantly decreased KCl-evoked changes in fluorescence by 14.9 ± 2.3% ($p<0.05$, $n = 9$) (Fig. 2.12b).

Bupropion-induced effects on Ca^{2+} fluxes in SH-SY5Y cells

To determine the concentrations of bupropion likely to interact directly with nAChRs, we examined the same concentrations of bupropion for their effects on nicotine- or KCl-evoked changes in fluorescence in SH-SY5Y cells (Fig. 2.13; Dajas-Bailador et al., 2002). Bupropion resulted in a concentration-dependent inhibition of nicotine-evoked increases in fluorescence with blockade of 24.8 ± 4.6%, 30.0 ± 2.9%, 51.1 ± 3.6% and 93.9 ± 0.8% ($p<0.05$, $n = 5$) at 0.1 µM, 0.3 µM, 1 µM and 10 µM bupropion, respectively (Fig. 2.13a). Pre-incubation with the general nAChR antagonist mecamylamine (10 µM) almost completely prevented the nicotine-evoked increase in fluorescence (94.4 ± 0.8% block, $p<0.05$, $n = 4$; Fig. 2.13a), confirming that nicotine exerted its effects through nAChRs. However, bupropion had no significant effect on increases in fluorescence evoked by KCl (Fig. 2.13b). In contrast, the VOCC blocker, cadmium chloride (100 µM), inhibited KCl-evoked increases in fluorescence by 89.8 ± 1.1% ($p<0.05$, $n = 4$; Fig. 2.13b). Data from the pre-incubation with bupropion on nicotine-evoked changes in fluorescence

in SH-SY5Y cells were fitted to the Hill Equation (see section 2.2.3.2) to give an IC_{50} value of 0.82 ± 0.10 µM bupropion (Fig. 2.13c).

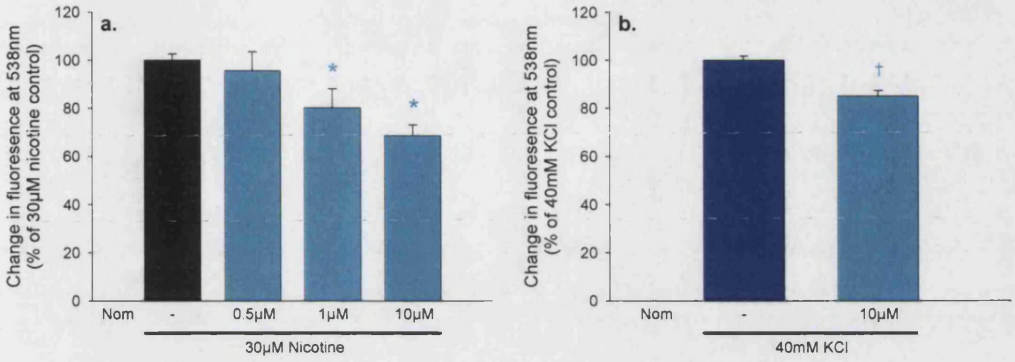


Fig. 2.12 Effect of nomifensine on nicotine- and KCl-evoked increases in fluorescence in SH-SY5Y cells.

SH-SY5Y cells were loaded with fluo-3 AM and incubated with or without nomifensine (Nom) for 10 min before stimulation with 30 µM nicotine or 40 mM KCl. Fluorescence changes at 20 s after addition of nicotine (a) or KCl (b) were measured and expressed as a percentage of the nicotine or KCl control response, measured in parallel in the absence of nomifensine. Values are the mean ± S.E.M. of at least four independent experiments, each carried out in quadruplicate ($^{\dagger}p<0.05$, $^{*}p<0.05$ significantly different from control response; unpaired Student's t-test, and one-way ANOVA with Dunnett's post-hoc test, respectively).

Combined effect of bupropion and nomifensine on Ca^{2+} fluxes in SH-SY5Y cells

The effect of bupropion (0.1-10 µM) in combination with 10 µM nomifensine on nicotine-evoked [^{3}H]DA release in rat striatal synaptosomes (Fig. 2.9a, b) and slices (Fig. 2.9c, d), illustrated a different effect as compared to that in the presence of bupropion alone (Fig. 2.8a, b). Here pre-incubation with increasing concentrations of bupropion (0.1-10 µM) in combination with 10 µM nomifensine were examined for their effect on nicotine- or KCl-evoked changes in fluorescence in SH-SY5Y cells (Fig. 2.14).

The collective effect of 10 µM nomifensine and bupropion exhibited concentration-dependent inhibition of nicotine-evoked increases in fluorescence by 41.6 ± 3.3%, 49.3 ± 4.7%, 69.9 ± 2.3% and 95.3 ± 0.4% ($p<0.05$, $n = 4$) at 0.1 µM, 0.3 µM, 1 µM and 10 µM bupropion, respectively (Fig. 2.14a). When the results were expressed as a percentage of the response to 30 µM nicotine in the presence of 10 µM nomifensine alone (Fig. 2.14b), bupropion maintained its concentration-dependent profile, exhibiting significant inhibition by 47.1 ± 4.2% and 91.9 ± 0.6% ($p<0.05$, $n = 4$) at 1 µM and 10 µM bupropion, respectively.

The combined effect of 10 µM nomifensine and bupropion on KCl-evoked changes in fluorescence, exhibited significant inhibition of KCl-evoked increases in fluorescence by 14.1 ± 4.9%, 20.2 ± 6.2%, 15.0 ± 5.4% and 21.2 ± 4.3% ($p<0.05$, $n = 4$), at 0.1 µM, 0.3 µM, 1 µM and 10µM bupropion, respectively (Fig. 2.14c). However, when the results were

expressed as a percentage of the response to 40 mM KCl in the presence of 10 µM nomifensine alone (Fig. 2.14d), on KCl-evoked changes in fluorescence, bupropion did not show any significant change in KCl-evoked increases in fluorescence.

Data from the co-incubation of 10 µM nomifensine and bupropion (0.1-10 µM) on nicotine-evoked changes in fluorescence in SH-SY5Y cells, were fitted to the Hill Equation (see section 2.2.3.2) to give an IC_{50} value of 0.24 ± 0.09 µM bupropion (Fig. 2.14e).

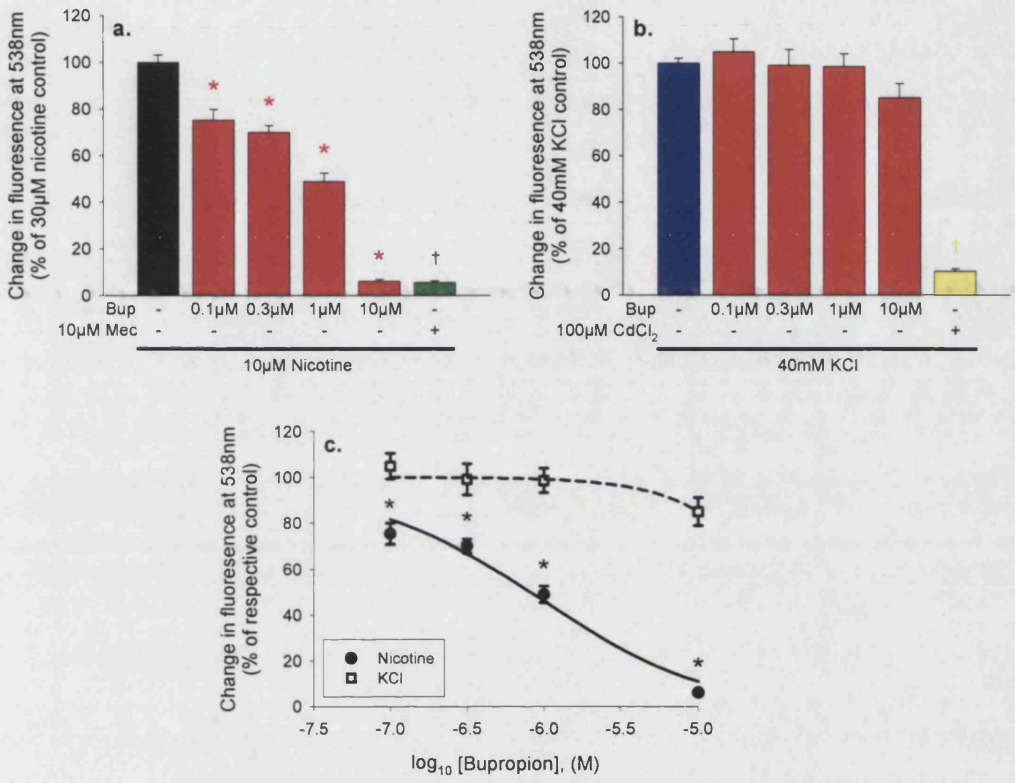


Fig. 2.13 Effect of bupropion on nicotine- and KCl-evoked increases in fluorescence in SH-SY5Y cells.

SH-SY5Y cells were loaded with fluo-3 AM and incubated with or without bupropion (Bup; 0.1-10 µM), mecamylamine (Mec; 10 µM) or cadmium chloride ($CdCl_2$; 100 µM) for 10 min before stimulation with 30 µM nicotine or 40 mM KCl. Fluorescence changes at 20 s after addition of nicotine (a) or KCl (b) were measured and expressed as a percentage of the nicotine or KCl control response, measured in parallel in the absence of bupropion, mecamylamine or cadmium chloride. Values are the mean ± S.E.M. of at least four independent experiments, each carried out in quadruplicate ($^{\dagger}p<0.05$, $^{*}p<0.05$ significantly different from control response; unpaired Student's t-test, and one-way ANOVA with Dunnett's post-hoc test, respectively). Concentration-response data were fitted to the Hill Equation, as described in section 2.2.3.2. The IC_{50} value for the inhibition by bupropion of nicotine-evoked increases in fluorescence (c) was 0.82 ± 0.1 µM.

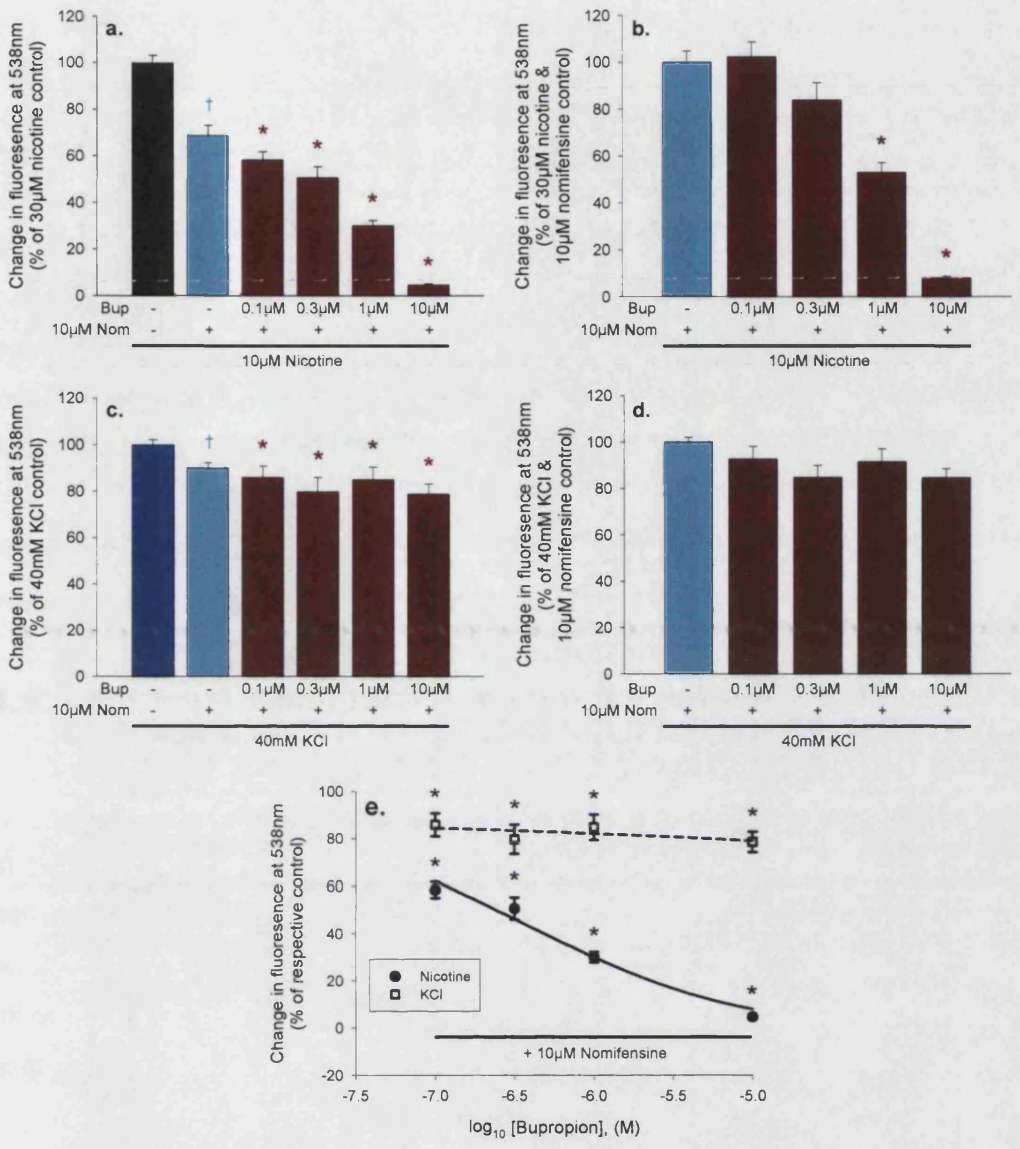


Fig. 2.14 Combined effect of bupropion and nomifensine on nicotine- and KCl-evoked increases in fluorescence in SH-SY5Y cells.

SH-SY5Y cells were loaded with fluo-3 AM and incubated with or without nomifensine (Nom; 10 μM) alone, or nomifensine (10 μM) in combination with bupropion (Bup; 0.1-10 μM) for 10 min before stimulation with 30 μM nicotine or 40 mM KCl. Fluorescence changes at 20 s after addition of nicotine (a, b) or KCl (c, d) were measured. Data are expressed as a percentage of the response to 30 μM nicotine in the absence (control; a) or presence (control; b) of 10 μM nomifensine, or 40 mM KCl in the absence (control; c) or presence (control; d) of 10 μM nomifensine, determined in parallel. Values are the mean ± S.E.M. of at least four independent experiments, each carried out in quadruplicate ($^{\dagger}p<0.05$, $^{*}p<0.05$ significantly different from control response; unpaired Student's t-test, and one-way ANOVA with Dunnett's post-hoc test, respectively). Concentration-response data were fitted to the Hill Equation, as described in section 2.2.3.2. The IC_{50} value for the inhibition by bupropion in combination with 10 μM nomifensine, of nicotine-evoked increases in fluorescence (e) was 0.24 ± 0.09 μM.

2.4 Discussion

Results presented in this chapter show that 10 μM bupropion decreases nicotine-evoked [^{3}H]DA release in both striatal synaptosomes and slices, consistent with the blockade of nAChRs. Furthermore, in slices but not in synaptosomes, bupropion produced a bell-shaped concentration-response, with inhibition of nicotine-evoked [^{3}H]DA release also observed in the presence of 0.1 μM bupropion. Reversal of the latter effect by the D2 antagonist raclopride leads us to propose that modest blockade of DAT by bupropion results in feedback inhibition via D2 receptors. This is overcome at higher concentrations of bupropion, before inhibition of nAChR occurs. Nomifensine, on the other hand, functions as a DAT blocker, but in the absence of DAT, may inhibit nAChRs and VOCC. These findings highlight the important role of *in vitro* neuronal models in dissecting drug pharmacology.

2.4.1 Validity of *in vitro* superfusion

The regulation of DA release by presynaptic nAChRs in brain preparations has been previously reported, using *in vitro* superfusion (Giorguieff-Chesselet et al., 1979; Kaiser et al., 1998; Wonnacott et al., 2000; Grady et al., 2002; Cui et al., 2003; Salminen et al., 2004). Further studies using this technique have revealed the presence of α6β2* (Klink et al., 2001; Champtiaux et al., 2003) and α4β2* nAChRs (Sharples et al., 2000; Klink et al., 2001; Champtiaux et al., 2003) on dopaminergic terminals in the striatum.

In the present study, brain preparations were in the form of synaptosomes and slices. Synaptosomes retain all features of the intact nerve ending and enable aspects of nerve terminal function to be studied in isolation; whilst slices retain local anatomical integrity, making the preparation more representative of the intact brain (see section 2.1.1.1).

Preliminary results (Fig. 2.5) indicate that nicotine-evoked [^{3}H]DA release from rat striatal synaptosomes was robust, reproducible and sensitive to mecamylamine, since nicotine-evoked [^{3}H]DA release above basal was almost totally abolished in the presence of the general nAChR antagonist (Fig. 2.5a). The concentration-response effect of nicotine on [^{3}H]DA release (Fig. 2.5b), which yielded an EC_{50} value of 4.8 ± 0.1 μM nicotine, was comparable to that obtained previously (Prince et al., 1996; Wonnacott et al., 2000), thereby confirming the reproducibility of the assay. Mecamylamine (10 μM) blocked a range of concentrations of nicotine-evoked [^{3}H]DA release (0.3-100 μM), but failed to do so at the highest concentration of nicotine (100 μM), thereby distinguishing nAChR-mediated and non-specific [^{3}H]DA release (Fig. 2.5c). Nicotine-evoked [^{3}H]DA release in striatal synaptosomes assayed by *in vitro* superfusion, yielded consistent results within

and between experiments, hence emphasising the reproducibility of the method. On this basis, a concentration-response curve for the effect of nicotine-evoked [^{3}H]DA release in striatal slices was not conducted. However, it would have enabled valuable comparisons to be made with the effect of nicotine-evoked [^{3}H]DA release in striatal synaptosomes.

2.4.1.1 Significance of [^{3}H]DA release profiles

The superfusion profiles showing the effects of nomifensine (Fig. 2.6) and bupropion (Fig. 2.7), on nicotine-evoked [^{3}H]DA release, were a particularly useful process of analysis. This method not only illustrated a difference in the effects of the drugs in synaptosomes and slices, by showing the variations in evoked and basal [^{3}H]DA release, but also illustrated drug effects throughout the course of the experiment. This characteristic was not evident in histograms, which quantitatively represented the effects of nomifensine and bupropion on nicotine-evoked [^{3}H]DA release (Fig. 2.8). Furthermore, normalising the effects of nomifensine (Fig. 2.6b, d) and bupropion (Fig. 2.7b, d) on nicotine-evoked [^{3}H]DA release, by expressing the results as a percentage of a fitted baseline, clarified any drug-induced effect on basal release. This was particularly important when evaluating any inhibitory actions on DAT provoked by either drug (discussed in section 2.4.2).

2.4.2 Inhibition of nAChR by DA reuptake blockers

Superfusion of synaptosomes results in the efficient removal of released neurotransmitter, due to the continuous flushing by the perfusing buffer flow. This minimises DA reuptake, and so application of bupropion produces only a transient increase in basal release (Fig. 2.7b). Hence blockade of DAT by bupropion is expected to have little discernable impact on the level of nicotine-evoked [^{3}H]DA release measured in these experiments (Fig. 2.15). The diminished response in the presence of increasing concentrations of bupropion (Fig. 2.8a) is in good agreement with the observations of Miller and colleagues (2002) who monitored the effects of bupropion on nicotine-evoked [^{3}H]DA release from striatal slices in the presence of nomifensine in the perfusing buffer, in order to eliminate any contribution from DAT blockade by bupropion (Miller et al., 2002). Indeed a similar inhibition of nicotine-evoked [^{3}H]DA release by 10 μM bupropion was observed in striatal slices in the present study (Fig. 2.8b).

The interpretation that inhibition of nicotine-evoked [^{3}H]DA release by bupropion reflects the drug's direct interaction with nAChRs is consistent with its selective inhibition of nicotine-evoked increases in fluorescence in SH-SY5Y cells (Figs. 2.13a, 2.16) and with previous studies (Fryer and Lukas, 1999; Slemmer et al., 2000; Miller et al., 2002).

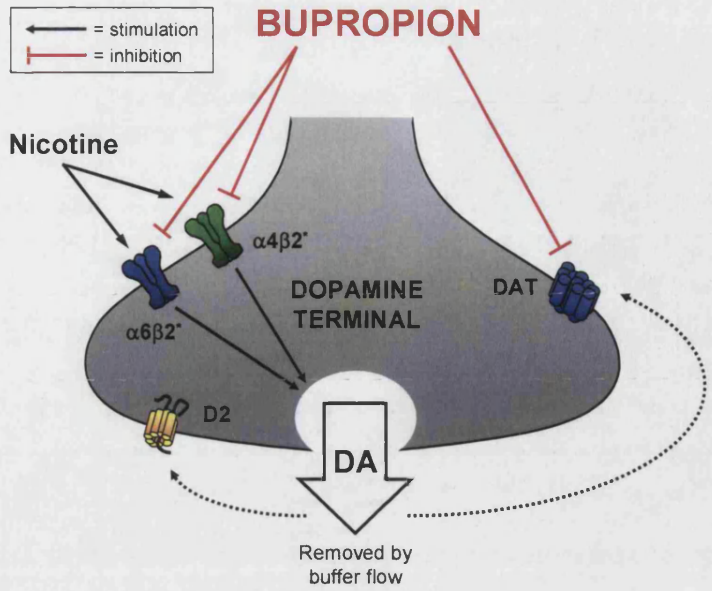


Fig. 2.15 Model of the actions of bupropion on nicotine-evoked DA release in striatal synaptosomes.

Nicotine induces DA release by stimulating α4β2* and α6β2* nAChRs on dopaminergic terminals. The continuous removal of released DA under the conditions of superfusion minimises DA reuptake by DAT, so that inhibition of DAT by low concentrations of bupropion has little impact. Similarly, DA does not accumulate to activate inhibitory D2-autoreceptors. At higher concentrations, bupropion inhibits the presynaptic nAChRs, reducing nicotine-evoked DA release.

Moreover, this blockade is specific to nAChR sites on dopaminergic terminals in the striatum, since bupropion inhibited nicotine-evoked [^{3}H]DA overflow in striatal slices, but did not inhibit [^{3}H]DA overflow, evoked by electrical field stimulation (Miller et al., 2002). Bupropion interaction with specific nAChR subtypes has been investigated using cell expression systems. Bupropion has been reported to inhibit Rb^+ efflux through α3β4 nAChR in SH-SY5Y cells with an IC_{50} value of 1.4 μM (Fryer and Lukas, 1999), in good agreement with the IC_{50} value of 0.8 μM for inhibition of nicotine-evoked increases in $[Ca^{2+}]_i$ in the same cell line (Fig. 2.13c). ACh-evoked currents in *Xenopus* oocytes expressing α3β2 and α4β2 nAChR were inhibited with IC_{50} values of 1.3 μM and 8 μM respectively (Slemmer et al., 2000). Failure to displace whole brain [^{3}H]nicotine binding sites meant that the inhibition was noncompetitive (Slemmer et al., 2000), although recent contrasting findings propose a competitive nature of bupropion inhibition using native α3β2* nAChRs (Miller et al., 2002). The subtypes of nAChR expressed on striatal DA terminals are heterogeneous, with α4,α5,α6 and/or β3 subunits combined with the common β2 subunit (Zoli et al., 2002; Champtiaux et al., 2003; Salminen et al., 2004; Cao et al., 2005). Their sensitivity to bupropion is similar to that of α4β2 nAChR, with approximately 65% inhibition by 10 μM bupropion (Fig 2.8; Miller et al., 2002). The difference in level of nAChR blockade by bupropion in synaptosomes and slices, as compared to that in SH-SY5Y cells, may be explained by the variation in composition of

nAChR subunit combinations that bupropion is likely to interact with: α4β2, α4α5β2, α6β2β3 and α4α6β2β3 in synaptosomes and slices (Wonnacott et al., 2005), versus α3β2, α3β4, α3α5β2, α3α5β4 and α3α5β2β4 in SH-SY5Y cells (Fryer and Lukas, 1999; Dajas-Bailador et al., 2002).

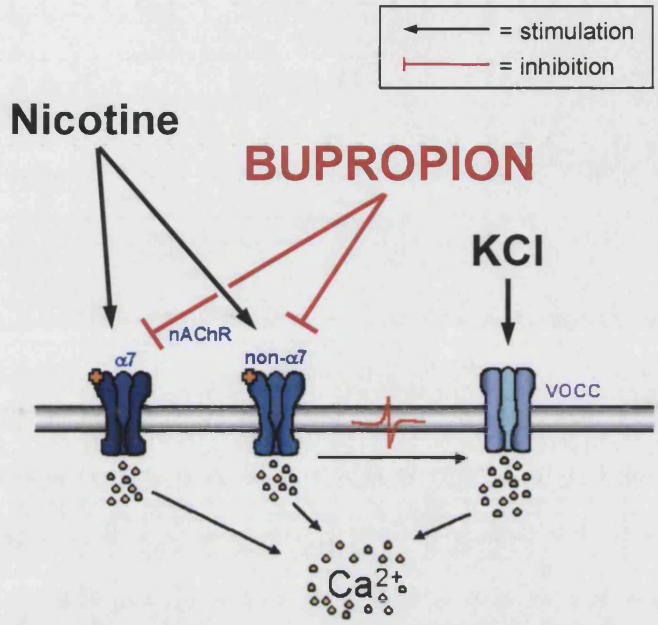


Fig. 2.16 Schematic representation of the actions of bupropion on $[Ca^{2+}]_i$ evoked by nicotine and KCl in SH-SY5Y cells.
SH-SY5Y cells express α7 and α3* nAChRs. In the absence of DAT, bupropion blocks nAChRs, thereby decreasing $[Ca^{2+}]_i$. It has no effect on VOCC.

It is understood that superfusion of synaptosomes minimises DA reuptake, but what could account for the significant inhibition of nicotine-evoked [^{3}H]DA release in the presence of nomifensine (Fig. 2.8a), a well defined DAT blocker? Since evoked release was calculated as the total radioactivity present under the peak, and as the percentage of mean basal release; the significant decrease in [^{3}H]DA release was attributed to the narrow peak in the presence of nomifensine, as compared to control (Fig. 2.6b). Nomifensine-induced inhibition of nicotine- (~ 30% block) and KCl-evoked (~ 15% block) increases in fluorescence in SH-SY5Y cells (Fig. 2.12), was indicative of nAChR and VOCC blockade, respectively, by 10 μM nomifensine (Fig. 2.17). Moreover, this level of inhibition of nicotine-evoked responses was comparable to the approximate 40% blockade of nicotine-evoked [^{3}H]DA release by nomifensine, in synaptosomes (Fig. 2.8a).

The additional inhibition of nicotine-evoked [^{3}H]DA release in synaptosomes (Fig. 2.9a, b), and nicotine- and KCl-evoked increases in fluorescence in SH-SY5Y cells (Fig. 2.14) following co-administration of bupropion and nomifensine, as compared to bupropion alone or nomifensine alone, may in part be attributed to the inhibition of nAChRs in synaptosomes and SH-SY5Y cells, by 10 μM nomifensine alone (Figs. 2.8a,

2.12), hence masking any effect of bupropion. The effects of bupropion in the combination treatments, which are similar to the effects of bupropion alone (Figs. 2.8a, 2.13a, b), are revealed when the results are expressed as a percentage of the response to 10 μM nicotine in the presence of 10 μM nomifensine alone (Figs. 2.9b, 2.14b, d).

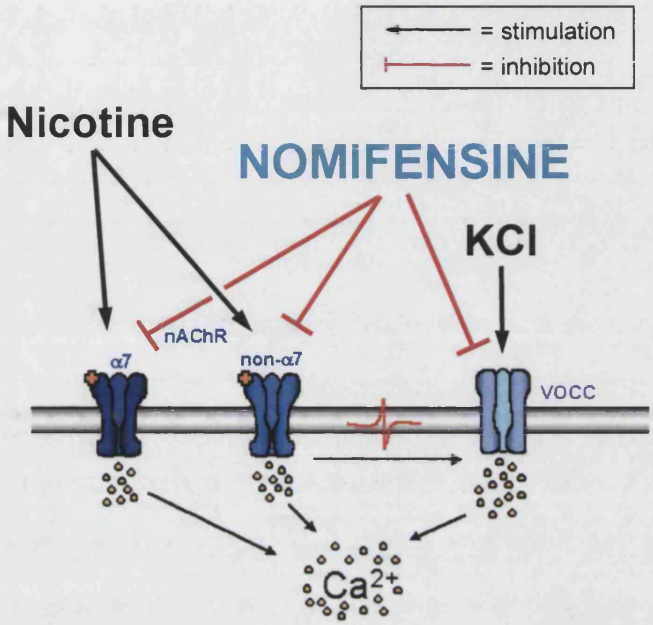


Fig. 2.17 Schematic representation of the actions of nomifensine on $[Ca^{2+}]_i$ evoked by nicotine and KCl in SH-SY5Y cells.
SH-SY5Y cells express α7 and α3* nAChRs. In the absence of DAT, nomifensine blocks nAChRs and VOCC, thereby decreasing $[Ca^{2+}]_i$.

Striatal slice preparations preserve some local anatomical relationships allowing the detection of striatal [^{3}H]DA release modulated by nAChR on neighbouring terminals, as well as by nAChR on DA terminals. In particular, α7 nAChR have been inferred to influence [^{3}H]DA release indirectly, through the release of glutamate (Kaiser and Wonnacott, 2000; Hamada et al., 2004). However, we can discount any interaction of bupropion with α7 nAChR in the present experiments as α7 nAChR have been shown to be much less sensitive to blockade by bupropion (IC_{50} = 60 μM, Slemmer et al., 2000; Alkondon and Albuquerque, 2005). This is consistent with the additivity of the inhibitory effects of 0.1 μM bupropion and 100 nM α-Bgt in response to 10 μM nicotine (Fig. 2.11b; relevance discussed in section 2.4.3.1).

2.4.3 Non-nAChR-mediated effects of bupropion

In contrast to the study of Miller and colleagues (2002), we did not inhibit DAT prior to addition of bupropion, in order to examine the relationship between DAT inhibition and

nAChR blockade by bupropion, with respect to nicotine-evoked [^{3}H]DA release in slice preparations. Somewhat surprisingly, a low concentration of bupropion (0.1 μM) decreased nicotine-evoked [^{3}H]DA release from slices (Fig. 2.8b), whereas blockade of DAT would be predicted to result in an increase in extracellular DA, as illustrated by 10 μM nomifensine (Fig. 2.8b). Reversal of this inhibition by raclopride (which had no effect on inhibition in response to 10 μM bupropion attributed to nAChR blockade; Fig. 2.10b) is compatible with a modest local increase in [^{3}H]DA due to partial DAT blockade, resulting in D2-mediated inhibition of [^{3}H]DA release, revealed as a net decrease in [^{3}H]DA levels measured. This is consistent with the disappearance of the effect at higher bupropion concentrations (0.3, 1 μM) that would afford a greater degree of DAT inhibition, producing higher levels of extracellular [^{3}H]DA that either mask the D2 inhibition or nullify it by activation of less sensitive D1 receptors. This interpretation is compatible with the potency of bupropion for blockade of DAT (IC_{50} = 0.6 μM; Hyttel, 1982; Damaj et al., 2004). However, the influence of 10 μM nomifensine for DAT inhibition (IC_{50} = 0.048 μM; Hyttel, 1982) produced a much higher level of extracellular [^{3}H]DA (Fig. 2.8b) than bupropion, and so would be more likely to mask any D2 inhibition and/or activate D1 receptors. This hypothesis is consistent with the concentration-dependent increase in nicotine-evoked [^{3}H]DA release following co-administration of bupropion and nomifensine in slices (Fig. 2.9c), where the effect of bupropion (0.1-10 μM) was masked by the influential effect of nomifensine.

Why was the decrease in nicotine-evoked [^{3}H]DA by 0.1 μM bupropion observed in slices but not synaptosomes? Superfusion is more effective at removing released transmitter from isolated nerve terminals than from slices, where some build up within the slice is expected to occur. Additionally, D2 receptors residing on neighbouring non-dopaminergic terminals could inhibit other transmitters to indirectly regulate DA release in slices. D2 receptors reside on glutamatergic terminals (Maura et al., 1988) where they may dampen the activity of subsets of corticostriatal inputs (Bamford et al., 2004). Moreover, endogenous corticostriatal glutamate release is modulated by D2 receptors only under conditions of stimulated and not basal release, suggesting that glutamate is not under tonic control by DA (Yamamoto and Davy, 1992). However, 0.1 μM bupropion, the concentration provoking the D2 inhibitory response, is insufficient to activate α7 nAChR (as revealed by the additivity of the inhibitory effects of 0.1 μM bupropion and 100 nM α-Bgt in response to 10 μM nicotine; Fig. 2.11b) and glutamate release (Fig. 2.11a). Therefore it is most probable that D2 inhibition of nicotine-evoked [^{3}H]DA release is via autoreceptors. This interpretation is consistent with interactions between D2-autoreceptors and DAT (Meiergerd et al., 1993), as D2-autoreceptors are also involved in controlling DA reuptake (Schmitz et al., 2002; Wu et al., 2002) during neurotransmission by partly compensating for the effects of DAT.

2.4.3.1 Complexity of DA-glutamate interactions in the striatum

The ability of ionotropic glutamate receptors to modulate striatal DA release has been reported in both *in vitro* (Wang, 1991; Desce et al., 1992) and *in vivo* studies (Sakai et al., 1997; Hernandez et al., 2003; Borland and Michael, 2004), and is compatible with evidence that glutamate can influence DA release via heteroreceptors on dopaminergic terminals (Wang, 1991; Desce et al., 1992). The degree of inhibition of nicotine-evoked [^{3}H]DA release seen with DNQX alone (Fig. 2.11a), an AMPA/kainate receptor antagonist, is comparable to that seen previously (Kaiser and Wonnacott, 2000), although in that report, [^{3}H]DA release was evoked by anatoxin-a, another nicotinic agonist. There is evidence that NMDA receptors also reside on striatal dopaminergic terminals and are capable of enhancing DA release (Desce et al., 1992). However, in that study, NMDA alone was ineffective, attributed to the Mg^{2+} block of NMDA receptors, whereas activation of AMPA/kainate receptors could overcome this inhibition. It has been proposed that ACh or (-)-nicotine, acting at presynaptic nAChRs on dopaminergic terminals, can relieve the Mg^{2+} block of NMDA receptors, to give a synergistic effect on DA release when (-)-nicotine and NMDA are co-applied in the presence of glycine (Cheramy et al., 1996), a co-agonist of NMDA receptors. Since our experiments were conducted in glycine-free conditions, blockade of NMDA receptors by AP-V alone (Fig. 2.11a), failed to affect nicotine-evoked [^{3}H]DA release, excluding the involvement of NMDA receptors. However, failure to observe an additive effect on the inhibition of nicotine-evoked [^{3}H]DA release following co-application of DNQX and 0.1 μM bupropion (Fig. 2.11a), together with the significant decrease in nicotine-evoked [^{3}H]DA release, when AP-V and 0.1 μM bupropion (Fig. 2.11a) were co-applied, was somewhat unexpected. Probable reasons for this may be attributed to ionotropic glutamate receptors being expressed on non-dopaminergic terminals in the striatum (Avshalumov et al., 2003); and possible involvement of mGluRs (Zhang and Sulzer, 2003) in regulating DA release in the striatum, thereby emphasising the complex network of DA-glutamate interactions.

In the present chapter, the inhibition of nicotine-evoked [^{3}H]DA release seen in the presence of α-Bgt alone (Fig. 2.11b), is comparable with previous work (Kaiser and Wonnacott, 2000), and consistent with the hypothesis that α7 nAChRs reside on striatal glutamatergic terminals to indirectly modulate DA release (Kaiser and Wonnacott, 2000). However, the added inhibition of nicotine-evoked [^{3}H]DA release following co-application of α-Bgt and 0.1 μM bupropion (Fig. 2.11b), is inconsistent with the presence of D2 receptors on glutamatergic terminals in the striatum, since co-application of raclopride and 0.1 μM bupropion (Fig. 2.10b) reversed the decrease in nicotine-evoked [^{3}H]DA release in the presence of 0.1 μM bupropion alone. However, since there are two types of cortical glutamatergic projections to the striatum: one being the pyramidal tract (PT) –type, which

contacts the indirect striatopallidal pathway neurones; and the second, referred to as the intratelencephalically projecting (IT) –type, which contacts the direct striatonigral pathway neurones (Lei et al., 2004), it is possible that D2 receptors may reside on the non-α7* nAChR-containing glutamatergic terminals that connect to the indirect pathway neurones (Fig. 2.18a), whilst the α7 nAChR-containing glutamatergic terminals that connect to the direct pathway neurones, are devoid of D2 receptors (Fig. 2.18b).

2.4.3.2 Putative model for the effects of 0.1 μM bupropion on nicotine-evoked [^{3}H]DA release in slices

The concentration of nicotine (10 μM) used in the present study is likely to activate both the direct and indirect pathway neurones (Hamada et al., 2004). Following nicotine-evoked [^{3}H]DA release, inhibition of DAT by 0.1 μM bupropion, increases extracellular [^{3}H]DA, which may enhance the effects of inhibitory D2-autoreceptors on dopaminergic terminals and possibly D2 receptors on neighbouring glutamatergic neurones, with a subsequent decrease in [^{3}H]DA release, providing a negative feedback loop in slices, but not in synaptosomes (Fig. 2.18a, b). Any interaction of [^{3}H]DA at D2 receptors on PT-type glutamatergic neurones would decrease the levels of glutamate released (via a non-α7* nAChR-mediated mechanism) for binding at ionotropic glutamate receptors on dopaminergic neurones, thereby decreasing the extracellular content of [^{3}H]DA (Fig. 2.18a).

Nicotine-evoked stimulation of α7 nAChRs on IT-type glutamatergic terminals would activate ionotropic glutamate receptors on dopaminergic terminals, with the release of glutamate. Interaction of [^{3}H]DA at D2-autoreceptors would dampen the activation of ionotropic glutamate receptors on dopaminergic terminals, thus decreasing [^{3}H]DA release (Fig. 2.18b). At the high concentration, bupropion (10 μM) would inhibit nAChRs and subsequently decrease nicotine-evoked [^{3}H]DA release, through the direct interaction with nAChRs on the dopaminergic terminals. Any effect of indirect modulation through the release of glutamate would not be prominent, since low quantities of [^{3}H]DA would be released in the first instance, and so would not be sufficient to activate the D2 receptor-mediated control from neighbouring terminals. On the contrary, the high levels of [^{3}H]DA produced following 10 μM nomifensine would mask any effect of D2 receptor modulation, due to the potency of nomifensine at DAT (see section 2.4.3). The gradual rise in nicotine-evoked [^{3}H]DA release with increasing concentrations of bupropion (0.1-1 μM) in slices (Fig. 2.8b), could be due to the increasing effect of bupropion as a DAT blocker, with an equilibrium being established between the direct and indirect modulation of [^{3}H]DA release. Any concentrations above 1 μM bupropion would produce nAChR inhibition, thereby significantly decreasing nicotine-evoked [^{3}H]DA release.

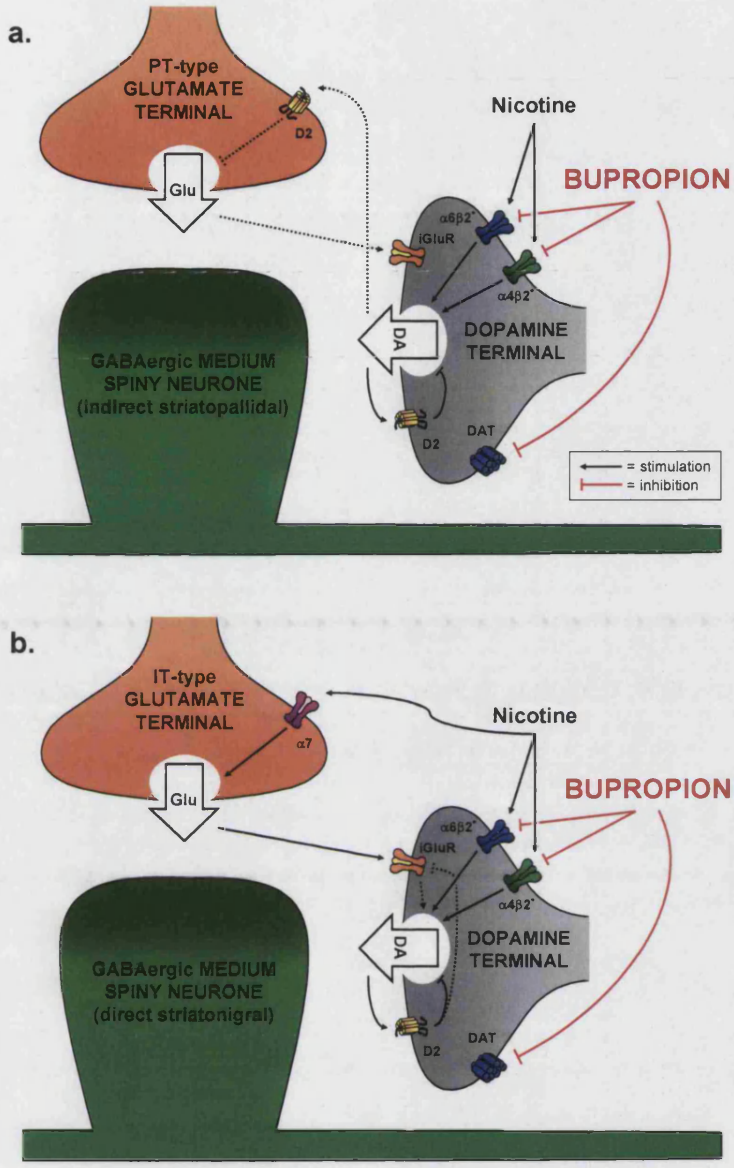


Fig. 2.18 Putative model of the actions of bupropion on nicotine-evoked DA release in striatal slices.

Dopaminergic and glutamatergic afferents converge onto the spines of the GABAergic neurones. Pyramidal tract (PT) –type glutamatergic projections contact the indirect striatopallidal pathway neurones (a) and intratelencephalically projecting (IT) –type glutamatergic projections contact the direct striatonigral pathway neurones (b). Nicotine also evokes DA release indirectly, by acting at α7 nAChR on neighbouring glutamatergic terminals, in addition to the stimulation of α4β2* and α6β2* nAChRs on dopaminergic terminals. Within the slice DA is removed less effectively and can be taken up by DAT. At low concentrations, bupropion inhibits DAT, which increases extracellular DA locally, leading to interaction with inhibitory D2-autoreceptors on dopaminergic terminals and possibly with inhibitory D2 receptors on neighbouring PT-type glutamatergic terminals. Reduced levels of glutamate (Glu) released from PT-type terminals for binding at ionotropic glutamate receptors (iGluRs) on dopaminergic neurones, contributes to the net decrease in extracellular DA (a). Glutamate released following nicotine-evoked stimulation of α7 nAChRs on IT-type glutamatergic terminals activates ionotropic glutamate receptors on dopaminergic afferents. Inhibitory D2-autoreceptors dampen the activation of ionotropic glutamate receptors on dopaminergic terminals, thereby decreasing DA release (b). At higher concentrations, bupropion inhibits α4β2* and α6β2* nAChRs, but not α7 nAChR, to reduce nicotine-evoked DA release.

The concept of indirect modulation of [^{3}H]DA release via glutamate secretion is only an example, since in reality, the situation will be further complicated by the fact that DA release in the striatum, can be influenced by a variety of neurotransmitters. Previous studies have demonstrated that activation of 5-HT2C receptors tonically inhibits striatal DA release (Di Matteo et al., 2004; De Deurwaerdere et al., 2004; Alex et al., 2005). However, where in the brain these receptors are localised is not yet known. Moreover, both 5-HT3 receptors, which co-localise with nAChRs on striatal nerve terminals (Nayak et al., 2000), and 5-HT4 receptors, selectively modulate DA exocytosis associated with increased DA neurone firing rate (De Deurwaerdere et al., 1997; Porras et al., 2002; Porras et al., 2003).

2.4.4 Implications for smoking cessation

The range of bupropion concentrations (0.1-10 μM) used in the present chapter is consistent with human serum concentrations of bupropion, which can rise to a peak near 0.5-1.0 μM after oral administration (Hsyu et al., 1997). If these concentrations correlate with local concentrations of bupropion in the brain, substantial inhibition of nAChR subtypes modulating DA release would not be anticipated to occur, whereas the complex behaviour attributed to DAT inhibition and D2 receptor activation would dominate the response. However, *in vivo* the scenario is made more complex by the biological activity ascribed to the major metabolite hydroxybupropion, which can reach 10-100 times the concentration of the parent compound in the plasma (Hsyu et al., 1997). The active isomer, (2S,3S)-hydroxybupropion, preserves the potency of the parent compound with respect to inhibition of DAT and blockade of nAChR (Damaj et al., 2004).

The present chapter demonstrates that by employing neuronal models of increasing complexity, it is possible to tease out the components underlying the action of bupropion on nicotine-evoked [^{3}H]DA release. Inhibition of DAT and blockade of nAChR appear to be separated with respect to bupropion concentration, and the latter action is likely to be less important at predicted concentrations of bupropion in the brain. The results from striatal slice preparations have also revealed an unexpected contribution of D2 receptors that can, to some extent, mitigate the effects of DAT inhibition. Thus, these comparative experiments provide some useful insights into the mechanisms underlying the efficacy of bupropion as an anti-smoking agent. Further investigation of the neurochemical interactions of nicotine and bupropion in more physiologically relevant models (see Chapter 3) will provide a better understanding of bupropion's mechanisms of action that facilitate smoking cessation.

CHAPTER 3

Actions of bupropion on nicotinic receptor-mediated DA release and locomotor activity

3.1 Introduction

Superfusion of the striatal preparations provided a valuable tool to dissect the *in vitro* pharmacology of bupropion and nicotine, with respect to DA release, in simple yet robust neuronal models of increasing complexity (Chapter 2). However, to obtain a better understanding of the physiologically relevant neurochemical interactions of these drugs, intact neuronal connections in the whole brain are required. Therefore, the effects of bupropion on nicotine-evoked DA release are best studied *in vivo*.

Early attempts to study neurotransmitter function were made using the cortical cup (1953) and push-pull (1961) techniques. Although these methods contributed a great deal to our knowledge of the chemical environment of the brain, one of their main drawbacks was that they involved an open system in which the brain came into direct contact with the perfusion fluid. One method which overcame this problem was the introduction of brain microdialysis (also known as intracerebral or intracranial dialysis). In this chapter, the interaction of bupropion with nicotine-evoked DA release is studied using *in vivo* microdialysis.

3.1.1 *In vivo* microdialysis

Microdialysis is a powerful and versatile sampling technique by which endogenous compounds are continuously monitored in the extracellular fluid of living organisms by means of an implanted hollow fibre constructed from dialysis membrane, known as the microdialysis probe (Westerink, 1995). In its simplest form, microdialysis provides a "snap shot" of the local environment revealing critical features of what a neurochemical system looks like before, during and after drug administration or other physiologically relevant stimuli (Zhang and Beyer, 2005). Microdialysis was first introduced as early as 1966 by Bito and colleagues with the concept of using a dialysis bag to collect samples from the brain. Delgado and colleagues (1972) later refined the technique by developing the first microdialysis probe in which a solution was slowly perfused through a dialysis bag and carried to a site immediately accessible to the experimenter. However, it was Ungerstedt

and colleagues (1974) who not only improved the design of the microdialysis probe by enlarging the surface area of the dialysis membrane thereby increasing the efficiency of analyte collection, but also coupled microdialysis sample collection to liquid chromatography and electrochemical techniques (Benveniste, 1989).

Microdialysis is now a firmly established method for monitoring neurotransmitter levels in the brains of conscious, freely moving animals. Moreover, it is also used to infuse drugs into specific brain regions through the microdialysis probe. This process is termed retro-dialysis or reverse dialysis. The microdialysis procedure typically involves a perfusion fluid (closely resembling endogenous cerebrospinal fluid) that is constantly pumped within a dialysis membrane of the probe, which is surgically implanted in the brain region of interest. The microdialysis probe acts in a similar manner to a blood capillary, sampling the interstitial environment by a process of diffusion along a concentration gradient towards and away from the probe (Fig. 3.1). Hence, the flow of the perfusion fluid allows small molecules to exchange across the dialysis membrane and the interior fluid, termed the dialysate, is removed and externally analysed. The dialysate contains various neurotransmitters and their metabolites, as well as metabolic precursors and waste products. However, because the dialysate is analysed externally, it can be subjected to a variety of chemical separation techniques, providing superb chemical resolution and sensitivity that allows unequivocal identification of neurotransmitter. The recovery of neurotransmitter and delivery of drugs via the microdialysis probe is dependent on a number of variables, including temperature, pH, molecular weight, shape and charge of the substance, surface area of the dialysis membrane, the flow of the perfusion fluid, the speed of diffusion of the substances through the extracellular fluid and, to a lesser extent, the properties of the membrane (see section A1 for further details on microdialysis; Benveniste, 1989; Bourne, 2003).

3.1.1.1 Advantages and disadvantages

Microdialysis offers several inherent advantages over other *in vivo* perfusion techniques. For instance, by employing stereotaxic surgery, microdialysis has high anatomical precision, enabling small, adjacent structures (such as the core and shell of the NAc) to be examined independently. Moreover, simultaneous sampling from multiple brain sites is made possible by implanting more than one probe in the same animal. This particular advantage allows the effects of chemical stimulation at both cell body and terminal regions of specific pathways in the brain to be measured. As the microdialysis probe can also be used to infuse drugs directly into specific brain regions, the effects of local drug application on neurochemicals in the selected brain regions can be monitored. Moreover, this method of drug delivery enables the effects of drugs that do not readily cross the BBB to be examined. The microdialysis probe is smaller than devices used for

cortical cup or push-pull techniques, and thus displaces a smaller area of tissue. The dialysis membrane provides a physical barrier between the perfusate and tissue thereby protecting the tissue not only from the turbulent flow of the perfusate but also from the high molecular weight substances, such as bacteria, which cannot cross the membrane. The perfusate flow rate is lower than that used in other perfusion techniques (typically 0.5-2.0 µl/min) and thus the amount of analyte depletion from tissue is less.

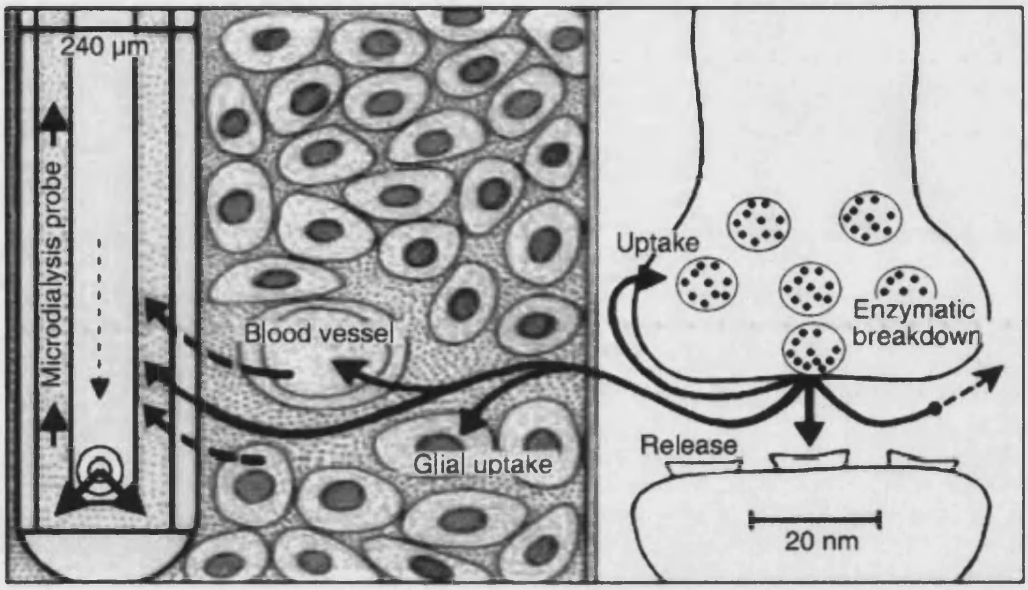


Fig. 3.1 Diagrammatic representation of passive transfer of endogenous substances into the microdialysis probe.
The dialysate flows down the inner of the two concentric tubes of the microdialysis probe and exits via a small hole at the bottom of the tube. The direction of flow is then reversed and the dialysate flows upwards, past the osmotic membrane, capturing substances that have diffused across the semi-permeable membrane, such as neurotransmitters and other low molecular weight substances (adapted from Bourne, 2003).

Despite many distinct advantages of microdialysis, it has a few disadvantages too. When microdialysis probes are implanted in a specific brain region, they cause damage to local tissue or cellular regions that could potentially confound experimental results. However, it should be noted that this damage is considered to be minimal and studies have shown that the BBB, for example, remains intact following probe implantation (Benveniste, 1989). Additionally, local chemical levels and blood flow return to normal levels approximately 24 h post probe implantation (Zhang and Beyer, 2005). Microdialysis also has the time delay of diffusion (tortuosity) of transmitter from the release site through a trauma or damage layer (caused by the large size of the probe) adjacent to the dialysis membrane (≥ 200 µm diameter dialysis tubing is typical). Microdialysis has poor temporal resolution and is impractical for dose-response studies. As samples are usually collected in time intervals greater than 5 min to provide sufficient sample for analysis, significant temporal integration and dampening of chemical dynamics render discrete phasic signals undetected.

Alternative to microdialysis

The only method that is currently an alternative to microdialysis is *in vivo* voltammetry, in which carbon fibre microelectrodes are inserted into selected brain sites. The small size of these microelectrodes as compared to microdialysis probes allow a much more localised region to be sampled than is possible with microdialysis. Diameters of carbon fibres typically range from 5-30 µm with lengths of 100-500 µm (Westerink, 1995). Voltammetry is excellent for identifying transient changes because it allows real-time monitoring of brain chemistry (on a scale of seconds, as opposed to minutes in microdialysis), thereby providing a higher temporal resolution than microdialysis. Voltammetry works by the ability of the working electrode (carbon graphite) to act as an oxidising (or reducing) agent, allowing the conversion of oxidisable agents to occur on the surface of the electrode (Bourne, 2003). Although this allows successful monitoring of catecholamines and their metabolites, and indoleamines, voltammetry is limited to the measurement of electroactive molecules only, thus exhibiting poor chemical resolution as compared to microdialysis. Moreover, other problems with voltammetry include the sensitivity and stability of signals obtained and the calibration of the microelectrodes. Voltammetry is much faster and samples from a smaller region, but is not as sensitive as microdialysis. However, the ultimate sensitivity of the microdialysis method is dependent on the species monitored and the subsequent methods of analysis.

3.1.1.2 Analysis of microdialysates

Microdialysis is dependent on sensitive analytical methods to measure compounds in the dialysate present at very low concentrations (fmol-nmol). Methods including fluorescence, ultraviolet and ECD, have been routinely coupled to HPLC for the measurement of neurotransmitter concentrations (see section A2 for a detailed explanation of HPLC with ECD). HPLC provides the advantages of simplicity and stability, making it possible to quantify amino acids, catecholamines, ACh and neuropeptides amongst other endogenous substances in the dialysates. As blood cells, proteins and other large molecules cannot enter the dialysate (due to the semi-permeable dialysis membrane) the samples are automatically cleaned up and injected, without further purification, into a HPLC system. By using flexible tubing to connect the microdialysis probe in the brain of the experimental animal to a HPLC system, and with the help of electronically controlled valves, a completely automated on-line microdialysis system can be achieved. An advantage of this approach is that results are available within minutes after sampling, thus allowing feed-back handling during the course of the experiment.

Measurement of analytes using HPLC is an indirect method. For example, quantification of catecholamines and their metabolites using HPLC with ECD (HPLC-ECD) is based on detection of neurotransmitter (or metabolite) oxidation at a carbon-based

electrochemical detector electrode following separation on an appropriate chromatographic column. Problems associated with this type of detection include spurious HPLC peaks, lengthy column equilibration time, poor HPLC peak shapes, and co-eluting interferences, which affect the overall accuracy of the measurements. Mass spectrometry provides a direct measurement of specific analytes (as detection is based on molecular weight of the compound) and, depending on the measured analyte, can provide a more sensitive platform to monitor some neurochemicals (Zhang and Beyer, 2005).

3.1.1.3 Microdialysis: fact or artefact?

A question raised frequently is whether neurotransmitters sampled by microdialysis reflect "true" synaptic release or a more unspecific overflow from synaptic and non-synaptic sources. This question has been addressed (Westerink, 2000) by examining the two classical criteria of neurotransmitter release: opening of fast Na^+ channels for the propagation of action potentials, and the Ca^{2+}-dependent exocytotic release of neurotransmitter. For the involvement of Na^{2+} channels, TTX is infused by reverse dialysis, whereas Ca^{2+} dependency is determined by either omitting Ca^{2+} from the perfusion fluid or by infusing Ca^{2+} channel blockers by reverse dialysis. Indeed, basal neurotransmitter output for the catecholamines and ACh have been shown to be TTX-sensitive and Ca^{2+}-dependent to an extent, indicative of neuronal transmission (Di Chiara, 1990). Microdialysis probes do not completely remove all the Ca^{2+} from the surrounding extracellular fluid and so a total inhibition of neurotransmitter release is difficult to achieve. However, an almost complete depletion of dialysate neurotransmitter level is achieved when Ca^{2+}-blocking ions such as Cd^{2+}, or Ca^{2+}-chealating agents such as EDTA, or high Mg^{2+} concentrations are included in the perfusion fluid (Westerink, 2000). In the case of amino acids, basal output has been reported to be TTX-insensitive and Ca^{2+}-independent, suggesting that their release is of non-neuronal (possibly glial) origin or independent of physiological neuronal activity (Di Chiara, 1990). The use of TTX in determining synaptic transmission is controversial because of its ubiquitous effects. To reflect this uncertainty, recovered concentrations of neurotransmitter are usually referred to as extracellular concentration, not release (Bourne, 2003). Extracellular levels of catecholamine metabolites are often difficult to interpret because processes such as synthesis, metabolism, conjugation and efflux rate all contribute to the steady state values of these metabolites. Therefore, the metabolites are slower indicators of changes in neuronal activity and are likely to represent more long-term changes in the release of catecholamines (Westerink, 1995).

It is generally believed that brain microdialysis does not directly measure neurotransmitter released at synaptic sites, but rather neurotransmitter that has diffused into the extracellular space. Hence, the final concentration of neurotransmitter sampled is

a result of processes such as reuptake, enzymatic degradation and tortuosity. Consequently, estimations (based on dialysate data or *in vitro* recoveries) of the absolute concentrations of neurotransmitter in the extracellular fluid are of little relation to its concentration in the synaptic cleft. Instead, the relative changes in dialysate neurotransmitter concentration are of greater value and are, therefore, usually expressed as a percentage of basals.

3.1.2 Aims of this chapter

Microdialysis studies in rodents have been fundamental in demonstrating the effects of nicotine on DA release in brain regions of the mesolimbic pathway (refer to section 1.4.6). Indeed, nAChR activation by acute systemic administration of nicotine in nicotine-naïve rats has been shown to stimulate DA transmission in most of these brain areas (see section 1.4.6.3). Microdialysis sampling of extracellular DA in the NAc_{core} and NAc_{shell} has also revealed the core/shell heterogeneity in response to chronic nicotine administration (see section 1.4.6.4). With respect to bupropion, brain microdialysis studies have shown that its ability to increase extracellular DA levels in the striatum and NAc after acute systemic and local application of bupropion in those particular brain regions is attributed to DAT blockade (see section 1.6.2.1).

Animal locomotor studies are commonly used to illustrate a DA-mediated behaviour. For example, the locomotor stimulant effects of nicotine are reduced following either 6-hydroxydopamine lesions, blockade of DA D1 and D2 receptors by their respective antagonists, or blockade of nAChRs in the VTA by DHβE, implicating a role of DA in the mesolimbic system for the locomotor stimulating effects of nicotine (Di Chiara, 2000). In chronically treated rats, nicotine-induced locomotor activity is increased in a dose-dependent manner up to a subcutaneous dose of 0.4 mg/kg. If a higher dose (0.8 mg/kg, s.c) is given, the animals fail to develop tolerance and the activity observed during the first 60 min after the injection, although higher than that observed for saline-treated controls, is lower than that recorded for rats treated chronically with 0.2 or 0.4 mg/kg nicotine (Clarke and Kumar, 1983). Indeed, bupropion administration has also been shown to dose-dependently increase locomotor activity in animals (see section 1.6.2.1). These hyperlocomotor effects of nicotine and bupropion are comparable to the increased extracellular DA levels in the mesolimbic brain areas observed in microdialysis studies.

It is clear from the results obtained in Chapter 2 that bupropion interacts with nAChRs to decrease nicotine-evoked [^{3}H]DA release in striatal synaptosomes and slices, and concentration-dependently inhibit nicotine-evoked increases in Ca^{2+} fluorescence in SH-SY5Y cells, consistent with nAChR antagonism. Comparable effects have been demonstrated *in vivo* by Slemmer and colleagues (2000), who showed that bupropion

dose-dependently blocks various nicotinic effects (see section 1.6.2.3). However, the neurochemical interactions of bupropion with nicotine-evoked DA release have not yet been examined *in vivo*. The present chapter aims to explore this relationship and to see if the nAChR-inhibiting property of bupropion observed in Chapter 2, translates *in vivo*. The neurochemical actions of nicotine and bupropion were examined using *in vivo* microdialysis, thereby increasing the level of complexity of the neuronal models employed in Chapter 2. Also, behavioural comparisons to these effects were made using locomotor activity tests.

Therefore, the particular aims of this chapter are:

- To explore the interactions of bupropion with nicotinic effects *in vivo*.
- Firstly, by defining the effects of bupropion on rat locomotor activity, to investigate any nAChR-blocking action of bupropion on nicotine-induced hyperlocomotion.
- Then, by optimising the concentrations of bupropion and nicotine for neurochemical comparisons, to extend the study by examining the effects of bupropion on nicotine-evoked DA release using *in vivo* microdialysis.

3.2 Materials and Methods

Animals

Male Sprague Dawley rats (250-300 g; University of Bath Animal House breeding colony) were used. Prior to the experiments, rats were housed in groups of four per cage in a temperature- and humidity-controlled environment with free access to food and water. Rats were kept on a 12 h light:dark cycle. All experiments carried out in the present study were within the guidelines of the Animals Scientific Procedures Act (1986) and approved by Her Majesty's Government Home Office.

3.2.1 Locomotor activity experiments

Experimental design

Using an appropriate dose of bupropion, obtained from the dose-response effect of systemic bupropion on rat locomotor activity (Experiment 1), the effects of systemic nicotine with or without bupropion pretreatment (Experiment 2) were examined for rat locomotor activity. On the day of the experiment, rats were transferred to the test room and placed randomly into individual clear-sided, perspex activity test cages (H. 185 mm x W. 265 mm x L. 425 mm) with 22 photocells equally spaced along the outer walls and a central light source. Rats were allowed to acclimatise to these conditions for 1 h.

Experiment 1

Following acclimatisation, rats were given an intraperitoneal (i.p.) injection of 10, 30 or 60 mg/kg bupropion or saline. Horizontal activity was scored automatically by infrared detection beams at 20 min intervals for 200 min after the injection.

Experiment 2

Following acclimatisation, rats were given an i.p. injection of 30 mg/kg bupropion or saline. After 20 min, rats were challenged with a subcutaneous (s.c.) injection of 0.4 mg/kg nicotine or saline. Horizontal activity was scored automatically by infrared detection beams at 20 min intervals for 180 min after the second injection.

Bupropion and nicotine were dissolved in 0.9% saline and administered in a volume of 1 ml/kg. The pH of all drug solutions was adjusted to pH 7.4 before administration. Drug doses are expressed as the free base.

Data analysis

In both experiments, mean activity counts for each treatment group were determined at 20 min intervals. All values are mean ± S.E.M. for n = 8 rats per treatment group. Any significant dose-response effects of bupropion on rat locomotor activity (Experiment 1) were analysed by a one-way ANOVA with a main factor of treatment (bupropion vs. saline) and a repeated measurement factor of time (13 bins of 20 min each). Statistical comparisons in experiment 2, were carried out by a two-way ANOVA with main factors of treatment (saline + nicotine vs. saline + saline, bupropion + saline vs. saline + saline, bupropion + nicotine vs. bupropion + saline) and a repeated measurement factor of time (13 bins of 20 min each). Where statistical significance was observed in any data set, a one-way ANOVA with post hoc Dunnett's test for multiple comparisons was used. A P value of <0.05 was considered statistically significant. All statistical procedures were performed using StatView (v 5.0.1) for Windows.

3.2.2 Microdialysis experiments

Surgery and microdialysis

Rats were anaesthetised with medetomidine (1 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.) and transferred to a stereotaxic frame (David Kopf, Topanga, USA) with the upper incisor bar set at -3.2 mm below the interaural line. Rats were maintained on a homoeothermic blanket at 37°C throughout. An incision was made to reveal bregma, from which all coordinates were taken. Holes were then drilled for four anchor screws, and another for unilateral placement of a concentric microdialysis probe (O.D. 0.30 mm) with 2 mm exposed Hospal membrane tip (manufactured in house) into the NAc_{core} (coordinates: anterior: +1.2 mm; lateral: +2.0 mm; ventral: -7.8 mm; (Paxinos and Watson, 1986). Using dental cement, the probe and a tether screw (Instech Soloman, UK) placed posterior to the probe, were secured in place, and the wound sealed. Anaesthesia was reversed using atipamezole (1 mg/kg, s.c.). Following surgery, rats were individually housed in circular chambers (I.D. 395 mm x H. 360 mm) with the microdialysis probe connected to a liquid swivel (Instech Soloman, UK) and a counter-balanced arm to allow unrestricted movement. Rats were allowed a recovery period of at least 16 h with food and water available *ad libitum* and probes were continuously perfused with artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 2.5 mM KCl, 1.18 mM $MgCl_2$, 1.26 mM $CaCl_2$, and 2.0 mM Na_2HPO_4, adjusted to pH 7.4 with 100 mM H_3PO_4) at a flow rate of 1.2 µl/min. After this period, the experiment started with the collection of samples from freely moving rats at 20 min intervals into 0.3 ml polypropylene sample vials (HPLC Technology, UK) containing 5 µl of perchloric acid (0.1 M). Drugs were delivered via the probe or by systemic administration (see experimental design section below).

DA analysis

Detection and subsequent quantification of DA in the dialysis samples involved the use of reverse-phase, ion-pair HPLC-ECD. Briefly, the method employed a Spherisorb (I.D. 100 x 2.1 mm; Higgins Analytical) reverse-phase column packed with 5 µm C_{18} silica based octa decyl silane (ODS) material. A Bischoff solvent delivery pump with a pulse dampener (PD-120625, Presearch Ltd, Herts., UK) was used to circulate mobile phase (100 mM NaH_2PO_4, 1 mM EDTA, 1 mM octane sulphonic acid, 12% methanol, pH 4.0) at a flow rate of 0.2 ml/min. The mobile phase was filtered through a 0.22 µm filter (Millipore, Bedford, USA) and degassed under vacuum. DA concentrations in the dialysate samples and standards (each 20 µl) were determined by injection onto the column via a refrigerated (4°C) Triathlon autosampler. The standards were prepared daily from a stock solution of DA (1 mM) in a 1:1 mixture of deionised water and 0.1 M perchloric acid, stored at 4°C. Antec-Intro (Leyden, Netherlands) electrochemical detector was used in conjunction with an Antec "wall-jet" design cell (VT-03). The cell employs a high density, glassy carbon working electrode (+0.60 V) combined with an Ag/AgCl reference electrode. The electrode signal was integrated using a PowerChrom data acquisition system (ADInstruments, Oxfordshire, UK). The detection limit for DA was 1 fmol on column (see section A2 for the optimisation of the HPLC system).

Optimisation of nicotine and bupropion concentrations

To achieve a local concentration of 10 µM nicotine, consistent with the *in vitro* experiments (see section 2.3.1.2), 100 µM nicotine was selected for local application into the NAc_{core}, on the basis that approximately 10% of drug crosses the dialysis membrane (see section A1.3). Hence, a preliminary experiment was conducted where rats received a local infusion of 100 µM nicotine into the NAc_{core}, via the microdialysis probe, by addition to the aCSF. Control rats were infused with aCSF only.

To obtain a detectable increase in dialysate DA in the NAc_{core} following nicotine administration, another preliminary experiment was conducted where rats received a local infusion of 3 mM nicotine by reverse dialysis. This concentration of nicotine was selected on the basis of previous work (Marshall et al., 1997). Control rats were infused with aCSF only.

To minimise the use of rats, two concentrations of bupropion (1, 100 µM) were infused locally into the NAc_{core} of the same rat over different time points. These concentrations were selected on the basis of a 10% bioavailability when administered by reverse dialysis (see section A1.3), thereby achieving a local concentration of 0.1 µM and 10 µM bupropion, respectively, since the *in vitro* studies revealed that 0.1 µM and 10 µM bupropion exhibited significant differences in nicotine-evoked [^{3}H]DA release (Fig. 2.8b). Therefore, a preliminary experiment was carried out where rats received local infusions of 1 µM bupropion followed by 100 µM bupropion with or without a recovery period. The

recovery period was defined as the time in between the administration of 1 µM and 100 µM bupropion, where no drug was given.

Experimental design

The effects of systemic bupropion (Experiment 3) and local bupropion, given via the microdialysis probe (Experiment 4), were compared with respect to DA release evoked by local administration of nicotine. For both experiments, rats were assigned to one of four treatment groups. One group was administered bupropion and nicotine; another group received bupropion alone; an additional group was given nicotine alone; and the final group had received vehicle only. Four basal samples were taken from each group, before rats were subjected to the microdialysis experiments.

Experiment 3

Bupropion was prepared as previously described for locomotor activity experiments and administered by i.p. injection. Nicotine was applied locally into the NAc_{core}, via the microdialysis probe, by addition to the aCSF. Rats received the drugs based on the following regime:

- Group 1: at t = 0 min, 30 mg/kg bupropion; at t = 40 min, 3 mM nicotine (for 20 min).
- Group 2: at t = 0 min, 30 mg/kg bupropion.
- Group 3: at t = 0 min, saline; at t = 40 min, 3 mM nicotine (for 20 min).
- Group 4: at t = 0 min, saline.

Experiment 4

Bupropion and nicotine were applied locally into the NAc_{core}, via the microdialysis probe, by addition to the aCSF. Rats received the drugs based on the following regime:

- Group 1: at t = 20 min, 1 µM bupropion (for 140 min); at t = 80 min, 3 mM nicotine and 1 µM bupropion (for 20 min); at t = 160 min, 100 µM bupropion (for 140 min); at t = 220 min, 3 mM nicotine and 100 µM bupropion (for 20 min).
- Group 2: at t = 20 min, 1 µM bupropion (for 140 min); at t = 160 min, 100 µM bupropion (for 140 min);
- Group 3: at t = 80 and 220 min, 3 mM nicotine (for 20 min);
- Group 4: untreated control rats received neither drug.

In both experiments dialysates were sampled for 280 min following basal sample collection. There were no artefacts associated with the procedure of changing from a syringe containing aCSF to a syringe containing aCSF plus drug.

Histology

After completion of the final experiment, rats were killed with a lethal dose of pentobarbital (i.p.) followed by cervical dislocation, and their brains rapidly removed and stored in 4% paraformaldehyde in phosphate buffer for a minimum of 5 days. Serial coronal sections (100 µm thick) were cut using a vibratome and histological verification of probe placement was confirmed with reference to a stereotaxic atlas (Paxinos and Watson, 1986). Data are reported only from animals where probe membranes were correctly positioned in the NAc_{core}. Fig. 3.2 depicts a typical location of the dialysis membrane in the NAc_{core}.

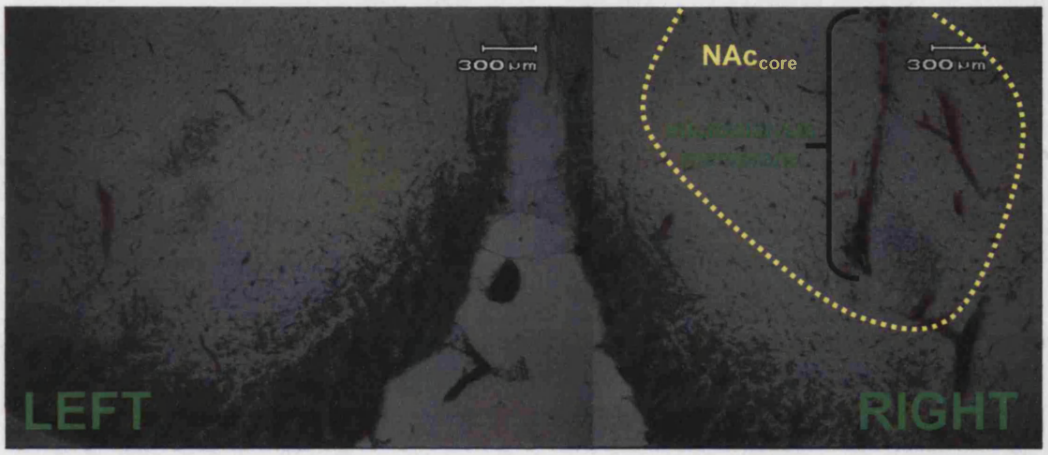


Fig. 3.2 Typical location of a microdialysis probe membrane in the NAc_{core}.
Probe tract can be seen in the right hemisphere, compared to the left (control) hemisphere, which had no surgical manipulation. NAc_{core} is outlined.

Data analysis

All data shown are mean ± S.E.M. values for at least n = 6 rats per treatment group. In all experiments, basal release measured in four consecutive samples before drug application was averaged and defined as 100%. Results for subsequent samples were calculated as percentages of this average basal release. Data from the optimisation experiments were analysed using a one-way ANOVA with main factors of treatment (100 µm nicotine vs. aCSF; 3 mM nicotine vs. aCSF; bupropion with recovery period vs. bupropion without recovery period) and a repeated measurement factor of time (18 bins of 20 min each). Data generated from experiments 3 and 4 were analysed using a two-way ANOVA with main factors of treatment (Experiment 3: bupropion + aCSF vs. saline + aCSF, bupropion + nicotine vs. saline + nicotine; Experiment 4: nicotine vs. aCSF, bupropion + nicotine vs. bupropion alone) and a repeated measurement factor of time (18 bins of 20 min each). In both experiments, to test the effect of drug against its own basal DA levels, repeated measures ANOVA were carried out with "time" as the "within-subjects" factor. Where statistical significance was observed in any data set, a one-way

ANOVA with post hoc Dunnett's test for multiple comparisons was used. A *P* value of <0.05 was considered statistically significant. All statistical procedures were performed using StatView (v 5.0.1) for Windows.

Drugs and reagents

(-)-Nicotine hydrogen tartrate, bupropion hydrochloride, DA and paraformaldehyde were purchased from Sigma-Aldrich Co. Ltd (Gillingham, Dorset, UK). All reagents used in HPLC analysis were of HPLC grade. EDTA, methanol, KCl, $MgCl_2$, Na_2HPO_4 and H_3PO_4 were purchased from Fisher Scientific Ltd (Loughborough, Leics., UK). NaCl, $CaCl_2$, NaH_2PO_4, octane sulphonic acid and perchloric acid were obtained from VWR International Ltd (Poole, Dorset, UK).

3.3 Results

3.3.1 Locomotor activity experiments

3.3.1.1 Locomotor effects of bupropion – Experiment 1

In order to establish a dose of systemically administered bupropion that was likely to evoke a significant detectable increase in extracellular DA in the NAc_{core}, in future microdialysis experiments, the effect of systemic bupropion (10, 30, 60 mg/kg, i.p.) was examined for rat locomotor activity, a DA-mediated behaviour (see section 3.2.1).

Although not significant, rats exhibited a brief period of activity following the saline injection (Fig. 3.3). Bupropion (10-60 mg/kg) increased rat locomotor activity, with 30 and 60 mg/kg bupropion producing a significant increase in locomotion ([$F(36, 336) = 8.476$, $p<0.0001$]), compared to saline-treated controls over a 200 min post-treatment period (Fig. 3.3). The maximal increase in locomotor activity was observed at 20 min following 30 mg/kg bupropion (+352 ± 91%, $p<0.001$, $n = 8$) and 60 mg/kg bupropion (+341 ± 64%, $p<0.001$, $n = 8$), compared to saline-treated controls. Locomotor activity remained significantly elevated for up to 80 and 200 min following 30 and 60 mg/kg bupropion, respectively.

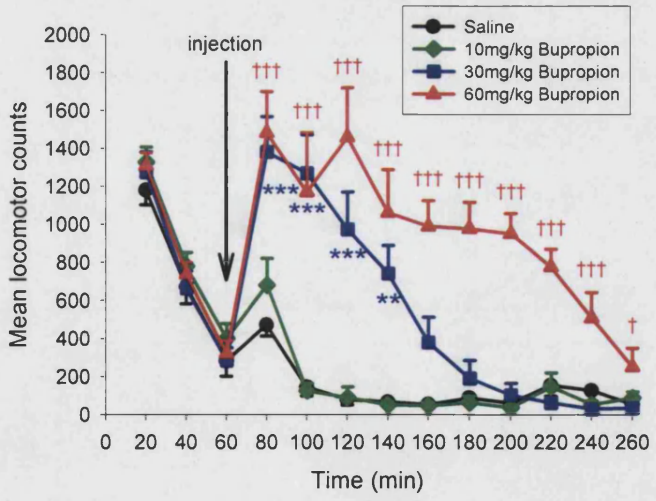


Fig. 3.3 Effect of bupropion on rat locomotor activity.
Rats were individually placed in activity test cages for a 60 min acclimatisation period, after which they were injected intraperitoneally with bupropion (10, 30, 60 mg/kg) or saline. Locomotor activity was recorded automatically by infrared detection beams at 20 min intervals. Data represent the mean ± S.E.M. locomotor counts, calculated from eight rats per treatment group. To test for a significant difference from saline-treated (control) rats, data were analysed using a one-way ANOVA with repeated measures and Dunnett's post-hoc test (**$p<0.01$, ***$p<0.001$ for 30 mg/kg bupropion vs. saline; $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$ for 60 mg/kg bupropion vs. saline). There was no significant difference in the activity after 10 mg/kg bupropion or between the different groups of rats during the acclimatisation period.

3.3.1.2 Locomotor effects of bupropion and nicotine in combination – Experiment 2

Rat locomotor activity was examined for the effects of bupropion on nicotine-induced hyperlocomotion. Using the median dose of bupropion from experiment 1 (30 mg/kg) because it produced hyperlocomotion followed by a steady-state decline in activity to saline-treated control levels, rats were pretreated with 30 mg/kg bupropion (i.p.) and challenged with an acute injection of nicotine (0.4 mg/kg, s.c.) after 20 min.

As in experiment 1, rats exhibited a brief period of activity following the saline injections (Fig. 3.4) but this failed to reach significance. Compared to saline-treated

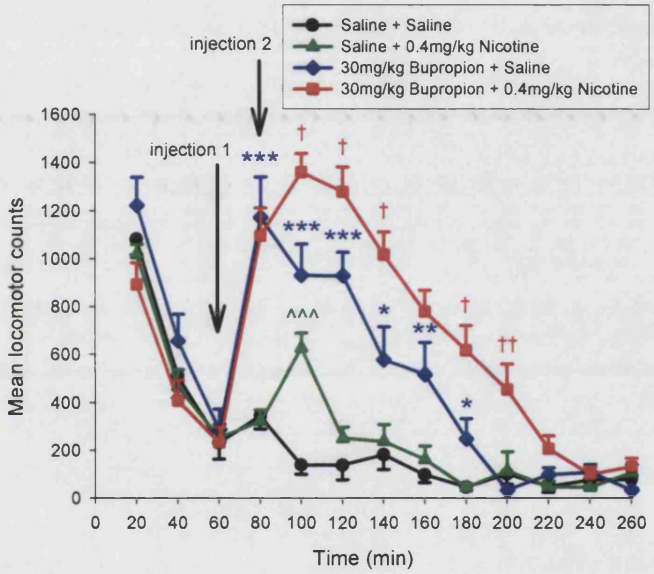


Fig. 3.4 Combined effect of bupropion and nicotine on rat locomotor activity.
Rats were individually placed in activity test cages for a 60 min acclimatisation period, after which they were injected intraperitoneally with 30 mg/kg bupropion or saline (injection 1). After 20 min, rats were challenged with a subcutaneous injection of 0.4 mg/kg nicotine or saline (injection 2). Locomotor activity was recorded automatically by infrared detection beams at 20 min intervals. Data represent the mean ± S.E.M. locomotor counts, calculated from eight rats per treatment group. To test for a significant difference from control rats, data were analysed using a two-way ANOVA with repeated measures and Dunnett's post-hoc test (^^^$p<0.001$ for Saline + 0.4 mg/kg Nicotine vs. Saline + Saline; *$p<0.05$, **$p<0.01$, ***$p<0.001$ for 30 mg/kg Bupropion + Saline vs. Saline + Saline; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ for 30 mg/kg Bupropion + 0.4 mg/kg Nicotine vs. 30 mg/kg Bupropion + Saline). There was no significant difference in the activity of the different groups of rats during the acclimatisation period.

controls, systemic nicotine significantly increased locomotor activity ([$F(1, 28) = 4.923$, $p<0.05$]), with peak locomotion observed at 20 min following the nicotine challenge (+917 ± 382%, $p<0.001$, $n = 8$). Nicotine-induced hyperlocomotion returned to saline-treated control values over the next 20 min (Fig. 3.4). In the absence of nicotine, bupropion significantly increased locomotion ([$F(1, 28) = 64.714$, $p<0.0001$]), compared to saline-treated controls over a 200 min post-bupropion period (Fig. 3.4). Maximal locomotor activity was observed at 20 min following bupropion pre-treatment (+402 ± 82%, $p<0.001$,

n = 8), compared to saline-treated controls. Post-bupropion, locomotor activity remained significantly elevated for up to 120 min. Compared to bupropion alone, nicotine significantly enhanced bupropion-induced hyperlocomotion (Fig. 3.4; [$F(12, 336) = 2.785$, $p<0.001$]). Optimal locomotor activity was observed at 20 min following the nicotine challenge (+170 ± 29%, $p<0.05$, n = 8), compared to bupropion-treated controls. Post-nicotine, locomotor activity remained significantly elevated for up to 120 min.

3.3.2 Microdialysis experiments

3.3.2.1 Optimisation of nicotine and bupropion concentrations

To investigate the effects of presynaptic nAChRs in the NAc_{core}, extracellular levels of DA were examined following local application of 100 µM nicotine in the NAc_{core}, by reverse dialysis. For the current preliminary experiment, the mean ± S.E.M. basal levels of DA were 50.2 ± 0.7 fmol/20 µl (n = 16). These data have not been corrected for recoveries across the dialysis membrane. Compared to aCSF controls, 100 µM nicotine (Fig. 3.5; t = 80-100 min, t = 220-240 min) failed to evoke DA release in the NAc_{core} ([$F(17, 119) = 1.509$, $p>0.05$]).

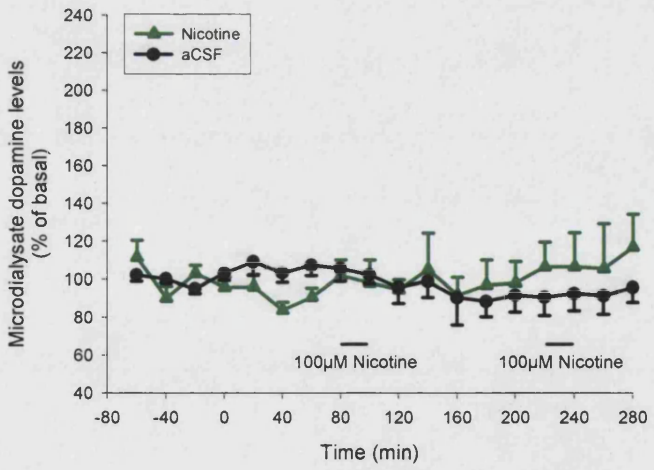


Fig. 3.5 Effect of local application of 100 µM nicotine on extracellular DA levels in the NAc_{core}.

Rats were prepared for microdialysis as described in section 3.2.2; 20 min (24 µl) serial fractions were collected and quantified for DA levels using HPLC with electrochemical detection. After collection of four stable baseline samples, rats were locally infused, via the microdialysis probe, with aCSF in the absence and presence of 100 µM nicotine. DA release is expressed as a percentage of mean ± S.E.M. basal release from at least seven rats per treatment group. To test for a significant difference from control (aCSF) response, data were analysed using a one-way ANOVA with repeated measures. Basal levels of DA were 50.2 ± 0.7 fmol/20 µl.

Since 100 µM nicotine was unsuccessful in increasing extracellular DA in the NAc_{core}, the effect of local infusion of 3 mM nicotine, via the microdialysis probe, was examined in the NAc_{core}. The concentration of nicotine (3 mM) infused by reverse dialysis was selected on the basis of previous work (Marshall et al., 1997), which showed a significant increase in DA levels following nicotine administration in the NAc, by reverse dialysis. For this preliminary experiment, the mean ± S.E.M. basal levels of DA were 55.6 ± 1.6 fmol/20 µl (n = 16). These data have not been corrected for recoveries across the dialysis membrane. Local infusion of 3 mM nicotine (Fig. 3.6; t = 80-100 min) in the NAc_{core}, significantly increased extracellular DA levels, compared to aCSF controls ([F(17, 187) = 5.964, p<0.0001]). Peak DA levels (t = 100 min) were 202 ± 34% (p<0.05, n = 7-9) above aCSF controls, and returned to basal values over the next 20-60 min (Fig. 3.6).

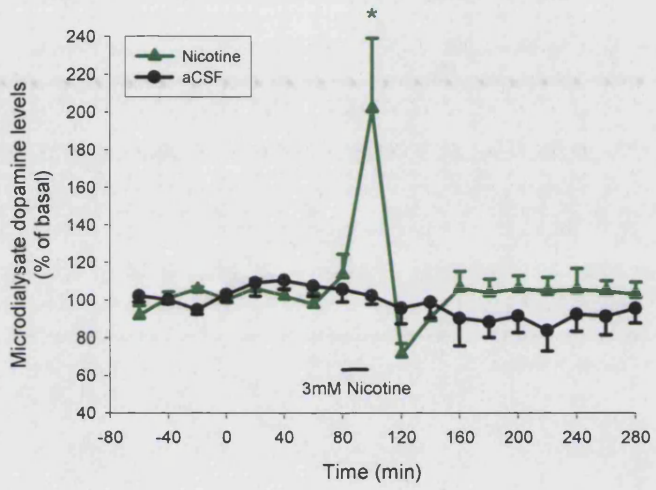


Fig. 3.6 Effect of local application of 3 mM nicotine on extracellular DA levels in the NAc_{core}.

Rats were prepared for microdialysis as described in section 3.2.2; 20 min (24 µl) serial fractions were collected and quantified for DA levels using HPLC with electrochemical detection. After collection of four stable baseline samples, rats were locally infused, via the microdialysis probe, with aCSF in the absence and presence of 3 mM nicotine. DA release is expressed as a percentage of mean ± S.E.M. basal release from at least seven rats per treatment group. To test for a significant difference from control (aCSF) response, data were analysed using a one-way ANOVA with repeated measures and Dunnett's post-hoc test (*p<0.05 for Nicotine vs. aCSF). Basal levels of DA were 55.6 ± 1.6 fmol/20 µl.

Rats, with bupropion (1, 100 µM) infused locally into the NAc_{core} by reverse dialysis, were compared for any changes in extracellular DA levels following bupropion administration with or without a recovery period. For this preliminary experiment, the mean ± S.E.M. basal levels of DA were 25.4 ± 1.0 fmol/20 µl (n = 14). These data have not been corrected for recoveries across the dialysis membrane. Extracellular DA levels in the NAc_{core} following local infusion of bupropion (1, 100 µM) administered with a recovery period (1 µM bupropion t = 20-140 min; 100 µM bupropion t = 240-360 min; Fig. 3.7) were not significantly different to those obtained when bupropion (1, 100 µM) was infused

without a recovery period (1 µM bupropion t = 20-140 min; 100 µM bupropion t = 160-280 min; Fig. 3.7; [F(17, 85) = 1.531, p>0.05]).

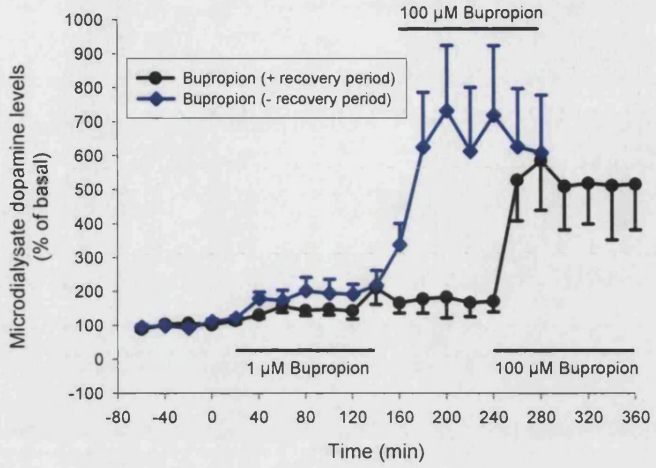


Fig. 3.7 Effect of locally administered bupropion on extracellular DA levels in the NAc_{core}.

Rats were prepared for microdialysis as described in section 3.2.2; 20 min (24 µl) serial fractions were collected and quantified for DA levels using HPLC with electrochemical detection. After collection of four stable baseline samples, rats were locally infused, via the microdialysis probe, with aCSF in the presence of 1 µM bupropion followed by 100 µM bupropion with or without a recovery period. DA release is expressed as a percentage of mean ± S.E.M. basal release from at least seven rats per treatment group. To test for a significant difference between each treatment measure, data were analysed using a one-way ANOVA with repeated measures. Basal levels of DA were 25.4 ± 1.0 fmol/20 µl.

3.3.2.2 Systemic effects of bupropion on nicotine-evoked DA release – Experiment 3

To investigate the effects of bupropion on nicotine-evoked DA release as a result of pharmacological interactions at both cell body and terminal regions of the mesolimbic dopaminergic pathway, bupropion (30 mg/kg, i.p.) was administered systemically followed by nicotine (3 mM), applied locally, via the microdialysis probe, in the NAc_{core}. The concentration of nicotine (3 mM) infused via the microdialysis probe was selected on the basis of preliminary experiments (see section 3.3.2.1) and previous work (Marshall et al., 1997). For this experiment, the mean ± S.E.M. basal levels of DA were 25.8 ± 0.4 fmol/20 µl (n = 37). These data have not been corrected for recoveries across the dialysis membrane.

In the absence and presence of nicotine, 30 mg/kg bupropion (Fig. 3.8a, b), significantly increased extracellular DA levels compared to basal values ([F(5, 17) = 6.598, p<0.0001]; [F(7, 17) = 17.569, p<0.0001], respectively). Peak DA levels (t = 60 min) in the absence and presence of nicotine were 113 ± 16% (p<0.01, n = 8) and 294 ± 50% (p<0.05, n = 9), respectively, above basal values. DA levels returned to basal values over

the next 20-40 min. Compared to saline-treated controls, bupropion significantly increased extracellular DA ([F(1, 24) = 8.774, p<0.01]) between t = 40-120 min (Fig. 3.8a). Compared to nicotine-treated controls, bupropion (Fig. 3.8b) enhanced nicotine-induced increases in extracellular DA ([F(17, 408) = 6.235, p<0.0001]). Raised DA levels were maintained between t = 40-60 min, with an average increase in DA of 122 ± 26% (p<0.05, n = 8-9) above nicotine-treated controls (Fig. 3.8b). Although not recorded as a criterion, rats that received bupropion (30 mg/kg, i.p.) with or without nicotine (3 mM) showed increased motor activity for a period of approximately 20-40 min, after being injected. The type of behaviour displayed was primarily grooming and investigating the surrounds of the microdialysis cage.

Local infusion of 3 mM nicotine (Fig. 3.8b; t = 40-60 min) in the NAc_{core}, significantly increased extracellular DA levels, compared to basal values ([F(6, 17) = 8.238, p<0.0001]), and compared to non-drug controls ([F(1, 24) = 5.506, p<0.05]). Peak DA levels (t = 60 min) were 126 ± 35% (p<0.01, n = 8) above basal values, similar to values observed previously (Fig. 3.6). DA levels returned to basal values over the next 20-40 min.

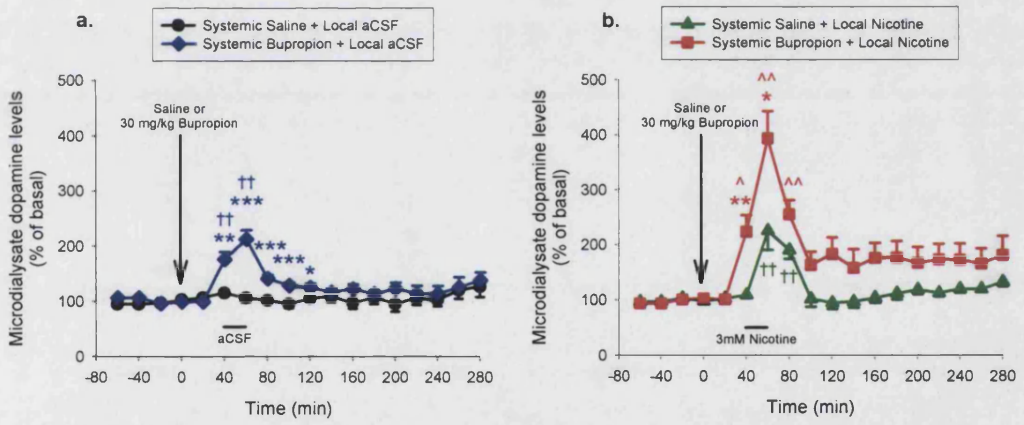


Fig. 3.8 Effect of systemic bupropion on nicotine-evoked DA release in the NAc_{core}. Rats, allocated to one of four treatment groups, were prepared for microdialysis as described in section 3.2.2; 20 min (24 µl) serial fractions were collected and quantified for DA levels using HPLC with electrochemical detection. After collection of four stable baseline samples, two groups of rats were injected intraperitoneally with saline or 30 mg/kg bupropion followed by a local infusion of aCSF, delivered via the microdialysis probe (a). Another two groups of rats were injected intraperitoneally with saline or 30 mg/kg bupropion followed by a local infusion of aCSF in the presence of nicotine, delivered via the microdialysis probe (b). DA release is expressed as a percentage of mean ± S.E.M. basal release from at least eight rats per treatment group. To test for a significant difference from basal values, data were analysed using repeated measures ANOVA and Dunnett's post-hoc test [(a) $^{\dagger\dagger}p<0.01$ for Systemic Bupropion + Local aCSF vs. basals; (b) $^{\dagger\dagger}p<0.01$ for Systemic Saline + Local Nicotine vs. basals, $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$ for Systemic Bupropion + Local Nicotine vs. basals]. To test for a significant difference from control response, data were analysed using a two-way ANOVA with repeated measures and Dunnett's post-hoc test [(a) $^{*}p<0.05$ $^{**}p<0.01$, $^{***}p<0.001$ for Systemic Bupropion + Local aCSF vs. Systemic Saline + Local aCSF; (b) $^{*}p<0.05$, $^{**}p<0.01$ for Systemic Bupropion + Local Nicotine vs. Systemic Saline + Local Nicotine]. Basal levels of DA were 25.8 ± 0.4 fmol/20 µl.

3.3.2.3 Local effects of bupropion and nicotine on extracellular DA levels – Experiment 4

In this experiment, both drugs were applied locally, via the microdialysis, probe to investigate the effects of bupropion-nicotine interactions within the NAc_{core}. Two concentrations of bupropion (1, 100 µM), administered without a recovery period (see section 3.3.2.1), were tested in the same rat over different time points for a total sample collection period of 360 min. For this experiment, the mean ± S.E.M. basal levels of DA were 37.9 ± 0.2 fmol/20 µl (n = 31). These data have not been corrected for recoveries across the dialysis membrane.

Local application of 3 mM nicotine in the absence of bupropion (Fig. 3.9a; t = 80-100 min, t = 220-240 min), significantly increased extracellular DA levels, compared to basal values ([F(6, 17) = 3.253, p<0.001]), and compared to non-drug controls ([F(17, 204) = 3.341, p<0.0001]). Peak DA levels (t = 120 and t = 260 min) were 56 ± 24% (p<0.05, n = 8) and 69 ± 29% (p<0.01, n = 8) respectively, above basal values. DA levels returned to basal values over the next 20 min.

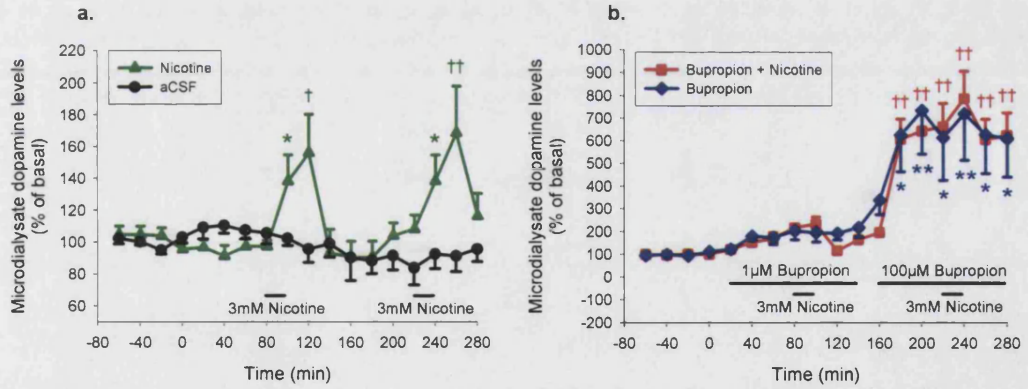


Fig. 3.9 Effect of locally administered bupropion and nicotine on DA release in the NAc_{core}.

Rats, allocated to one of four treatment groups, were prepared for microdialysis as described in section 3.2.2; 20 min (24 µl) serial fractions were collected and quantified for DA levels using HPLC with electrochemical detection. After collection of four stable baseline samples, rats were locally infused, via the microdialysis probe, with aCSF in the absence and presence of 3 mM nicotine (a) or 1 µM and 100 µM bupropion in the absence and presence of 3 mM nicotine (b; note increased scale on y-axis). DA release is expressed as a percentage of mean ± S.E.M. basal release from at least seven rats per treatment group. To test for a significant difference from basal values, data were analysed using repeated measures ANOVA and Dunnett's post-hoc test [(a) $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ for Nicotine vs. basals; (b) $^{\dagger\dagger}p<0.01$ for Bupropion + Nicotine vs. basals, $^{*}p<0.05$, $^{**}p<0.01$ for Bupropion vs. basals]. To test for a significant difference from control response, data were analysed using a two-way ANOVA with repeated measures and Dunnett's post-hoc test [(a) $^{*}p<0.05$ for Nicotine vs. aCSF]. Basal levels of DA were 37.9 ± 0.2 fmol/20 µl.

In the absence of nicotine, 100 µM bupropion (Fig. 3.9b) significantly increased extracellular levels of DA compared to basal values ([F(3, 17) = 13.850, p<0.0001]), but 1

μM bupropion (t = 20-140 min) failed to produce any significant elevation. Following 100 μM bupropion, raised DA levels were maintained between t = 180-280 min, with an average increase in DA of 554 ± 23% ($p<0.05$, n = 7) above basal values. Compared to non-drug controls, both 1 μM and 100 μM bupropion increased extracellular levels of DA ([$F(6, 72) = 4.409$, $p<0.001$]; [$F(6, 66) = 6.038$, $p<0.0001$], respectively). Although not recorded as a standard measure, rats infused with 100 μM bupropion, but not 1 μM bupropion, with or without nicotine (3 mM) displayed heightened motor activity, similar to that seen in experiment 3 following systemic bupropion (30 mg/kg). The increased behavioural response was maintained during infusion of 100 μM bupropion (t = 180-280 min). There was no main effect of treatment, following nicotine co-application (Fig. 3.9b) with either 1 μM bupropion ([$F(6, 60) = 1.742$, $p>0.05$]) or 100 μM bupropion ([$F(6, 66) = 0.537$, $p>0.05$]). Even though 1 μM bupropion and nicotine (t = 20-140 min) failed to induce any significant rise in extracellular DA, 100 μM bupropion and nicotine (Fig. 3.9b) significantly increased extracellular levels of DA ([$F(5, 17) = 23.919$, $p<0.0001$]), compared to basal values. The average increase in DA (t = 180-280 min) was 554 ± 28% ($p<0.01$, n = 7) above basal values.

3.4 Discussion

The main objective of the present chapter was to probe the putative antagonistic actions of bupropion upon *in vivo* effects of nicotine in rats. Bupropion failed to exhibit any efficacy as a nAChR antagonist. Moreover, the weak DA reuptake blocking actions of bupropion were inferred to explain some of the augmented effects of nicotine produced by this antidepressant. Unlike conventional nAChR antagonists, such as mecamylamine or DHβE, that typically decrease nicotine-induced activation of dopaminergic effects (Marshall et al., 1997; Grottick et al., 2000), bupropion pre-treatment slightly enhanced nicotine-evoked DA release (Fig. 3.8) and nicotine-induced hyperlocomotion (Fig. 3.4), which was consistent with an absence of bupropion effect as a nicotinic antagonist.

3.4.1 *In vivo* microdialysis: methodological aspects

Local infusion of bupropion in the NAc_{core} without a recovery period was selected as the appropriate paradigm, as similar levels of extracellular DA were obtained when bupropion was infused with or without a recovery period (Fig. 3.7). The significant increase in extracellular DA levels observed following local infusion of 100 µM bupropion (Fig. 3.9b) was in line with previous work (Nomikos et al., 1990). However, in that study, bupropion (100 µM) was infused locally in the rat striatum, which encompasses the caudate-putamen and NAc_{core} (see section 1.4.5.2; Voorn et al., 2004). With a K_i of 0.6 µM for DA reuptake (Hyttel, 1982; Richelson and Pfenning, 1984) and assuming a 10% bioavailability of bupropion when given by reverse dialysis (see section A1.3; 1 µM bupropion infusion = 0.1 µM extracellular bupropion), it is not surprising that 1 µM bupropion (Fig. 3.9b) did not significantly elevate extracellular DA. At 1 µM, there is little possibility of sufficient bupropion being present in the NAc_{core} to block DAT. Furthermore, the distance for bupropion to diffuse within the NAc_{core}, after passing through the dialysis membrane, may also influence the result.

Since acute systemic administration of nicotine in rats that are naïve to the alkaloid, does not increase dialysate DA in the NAc_{core} (Nisell et al., 1997; Di Chiara, 2000; Iyaniwura et al., 2001), the present study measured dialysate DA levels from the NAc_{core} following local infusions of nicotine (Figs. 3.8b, 3.9a), via the microdialysis probe. This method permitted the stimulation of presynaptic nAChRs in the NAc_{core}, without any contribution from nAChRs in the cell body regions of the mesolimbic dopaminergic pathway. As local infusion of 100 µM nicotine in the NAc_{core} failed to increase extracellular DA (Fig. 3.5), 3 mM nicotine was selected for local application, via the microdialysis probe. The rise in extracellular DA levels observed after the local application of 3 mM nicotine (Figs. 3.6, 3.8b, 3.9a) was consistent with previous work (Marshall et al., 1997).

However, in that study, nicotine (3 mM) was infused locally in the NAc, without differentiating between NAc_{core} and NAc_{shell}. The study also revealed that at 3 mM nicotine, evoked increase in DA release was abolished by the prior administration of mecamylamine, via the microdialysis probe, confirming the mediation of nAChRs (Marshall et al., 1997). Two successive local applications of 3 mM nicotine in the NAc_{core} (Fig. 3.9a) gave similar increases in extracellular DA (56 ± 24%, 69 ± 29% above basal values) suggesting that there was no evidence of an elevation or reduction in DA efflux after the second nicotinic challenge. Confirmation that nicotine-evoked increases in extracellular DA returned to baseline levels was depicted (Fig. 3.6).

3.4.2 Differences in pharmacological interactions of nicotine and bupropion: systemic vs. local administration

Systemic administration of drug, compared to local application of drug, closely resembles a more physiological state, since the former allows for cell body regulation of neuronal firing and neurotransmitter efflux (Ferrari et al., 2002). Hence, the effects of systemic bupropion (Figs. 3.3, 3.4, 3.8a, b) or nicotine (Fig. 3.4) would be under cell body control, whilst those of local bupropion (Fig. 3.9b) or nicotine (Figs. 3.8b, 3.9a) in the NAc_{core}, would not. This can explain why the effects of systemic bupropion and local application of nicotine in the NAc_{core} (Fig. 3.8b), were significantly different to those of systemic bupropion in the absence of local nicotine (Fig. 3.8a), whereas, the combination of bupropion and nicotine applied locally into the NAc_{core} (Fig. 3.9b), failed to show any significant difference to the effects of bupropion in the absence of nicotine (Fig. 3.9b).

It is interesting to note that bupropion evoked a transient increase followed by a swift decline (similar to the effects of a releasing agent) in extracellular DA when administered systemically (Fig. 3.8a, b), compared to a sustained increase (similar to the effects of a reuptake inhibitor) when applied locally in the NAc_{core} (Fig. 3.9b). A possible explanation lies in understanding the pharmacokinetics of bupropion in rats. Bupropion induces its own metabolism (Schroeder, 1983) and the major metabolites are hydroxybupropion, threohydrobupropion and erythrohydrobupropion (Hsyu et al., 1997). Bupropion is widely distributed among rat tissues with brain having a tissue:plasma ratio of approximately 28:1, 1 hr post-dose (Schroeder, 1983). Bupropion has a short half-life in rats ($t_{½}$ = 26 min; Suckow et al., 1986) and rapid inhibition of DAT binding occupancy rate in the striatum (1 min; Stathis et al., 1995). Therefore, following systemic administration (Fig. 3.8a, b), the short half-life of bupropion (30 mg/kg) would limit its occupancy of DAT before being metabolised, which would subsequently limit its effect in increasing

extracellular DA levels via DAT inhibition. Furthermore, systemic administration of bupropion would mean that the concentrations of bupropion reaching the NAc_{core} would be divided amongst various other brain regions comprising DAT, as well as other organs of metabolism and excretion. Therefore, the increase in extracellular DA and subsequent raised baseline following systemic bupropion (Fig. 3.8a, b) would be attributed to DAT blockade of dopaminergic neurones in the mesolimbic pathway, as opposed to just the NAc_{core}. The sustained increase in extracellular DA following local application of 100 µM bupropion (Fig. 3.9b) was due to stable levels of bupropion being maintained throughout the infusion period (t = 160-280 min), which allowed for a steady state effect of bupropion. Moreover, local application meant that bupropion was delivered directly into the NAc_{core}, thereby substantially circumventing the pharmacokinetic factors that occur after systemic administration.

Elevated rat locomotor activity observed after systemic administration of bupropion and nicotine (Fig. 3.4), compared to bupropion alone (Fig. 3.4) is comparable to the enhanced extracellular DA levels seen in the microdialysis experiments following systemic administration of bupropion and local application of nicotine in the NAc_{core} (Fig. 3.8b). However, the pharmacological actions responsible for these effects may be different as nicotine was administered systemically in the locomotor activity experiments (Fig. 3.4) and locally in the NAc_{core}, in the microdialysis experiments (Fig. 3.8b). Additionally, bupropion-induced potentiation of nicotine-evoked DA efflux (Fig. 3.8b), and locomotor activity (Fig. 3.4), cannot be a pharmacokinetic effect on the half life of bupropion, since the same effect was observed when nicotine was infused locally into the NAc_{core} (Fig. 3.8b), and systemically (Fig. 3.4).

3.4.3 Behavioural comparisons to the neurochemical interactions of nicotine and bupropion

Although relative increases in extracellular neurotransmitter levels measured by microdialysis are important, they provide limited support for the functional consequences of such changes *in vivo*. To obtain a handle on this latter issue, rat locomotor activity was measured as an index of enhanced limbic dopaminergic function (Rowley et al., 2000). The magnitude and time-course of bupropion-induced hyperlocomotion were dose-related (Fig. 3.3). Bupropion, at doses above 10 mg/kg, resembles psychomotor stimulants in increasing locomotor activity (Nielsen et al., 1986; Redolat et al., 2005). Moreover, at 30 mg/kg, bupropion has been shown to generalise to d-amphetamine in drug discrimination studies (Heal et al., 1992). However, another study revealed an absence of psychostimulant-like effect of bupropion (Shoaib et al., 2003), when tested for nicotine discrimination. A maximal effect in locomotion following 30 and 60 mg/kg bupropion was

probably due to rats displaying stereotypy after a certain level of locomotor activity was achieved (Cooper et al., 1980). Acute administration of 30 mg/kg bupropion (Fig. 3.3) resulted in a significant increase in locomotor activity over a period of 80 min post-injection. In contrast, extracellular levels of DA in the NAc_{core} were significantly elevated over a period of only 40 min (Fig. 3.8a) post-injection. Similar dissociations between stimulant-induced behaviours and increases in interstitial DA concentrations have been reported previously (Nomikos et al., 1989; Nomikos et al., 1992). It raises the question as to whether rapidly occurring postsynaptic adaptive mechanisms exist that compensate for the increased extracellular levels of DA in the NAc_{core}.

Systemic injections of nicotine activates nAChRs both in the cell body (VTA) and terminal regions (NAc and PFC) of the mesolimbic DA pathway, whilst local application of nicotine in the NAc_{core}, will activate nAChRs in the NAc_{core} only (Di Chiara, 2000; Ferrari et al., 2002). The locomotor stimulant property of nicotine appears to be initiated by nAChR activation within the VTA (Corrigall et al., 1994; Panagis et al., 1996) resulting in increased DA release in the NAc (Nisell et al., 1994b). Furthermore, DA release in the core subregion of the NAc, rather than shell, contributes preferentially to the locomotor stimulant effects of nicotine (Boye et al., 2001). Knockout studies have suggested a role for the $\alpha3$ and $\beta4$ subunits in mediating nicotine-induced hyperlocomotion (Salas et al., 2004), with $\alpha7$ nAChRs having a negligible role (Grottick et al., 2000). However, the $\alpha3$ and $\beta4$ subunits, which are thought to co-assemble with the $\alpha5$ subunit, are only expressed at relatively high levels in the habenular/interpeduncular nucleus system (Salas et al., 2004). Subunit compositions $(\alpha4)_2\alpha5(\beta2)_2$ and $\alpha4\alpha6\alpha5(\beta2)_2$ nAChRs have been proposed to reside on dopaminergic cell bodies (Klink et al., 2001) in the ratio 80:20 (Champtiaux et al., 2003). However, it is not known if these are segregated to distinct populations of dopaminergic neurones. In the terminal fields, four populations of nAChRs have been defined to exist on dopaminergic neurones, namely $(\alpha4)_2(\beta2)_3$, $(\alpha4)_2\alpha5(\beta2)_2$, $(\alpha6)_2(\beta2)_2\beta3$ and $\alpha4\alpha6(\beta2)_2\beta3$ nAChRs (Wonnacott et al., 2005). Together, these studies establish multiple loci through which nicotine might exert its effects when administered systemically (Fig. 3.4) compared to locally (Figs. 3.8b, 3.9a).

3.4.4 Putative mechanisms underlying bupropion's mode of action

Previous findings report that systemic and local effects of bupropion in the striatum are action potential-dependent, as they are attenuated by TTX (Nomikos et al., 1989; Nomikos et al., 1990). Additionally, bupropion does not affect monoamine oxidase activity (Ferris et al., 1982; Ascher et al., 1995). Hence, bupropion-induced increases in extracellular DA (Figs. 3.8a, b, 3.9b) and locomotor activity (Figs. 3.3, 3.4) may be

attributed to its ability to block DAT. However, the effects of bupropion on inhibitory autoreceptors or regulation of DA synthesis are unclear.

Re-uptake of DA by DAT, rather than metabolism or diffusion, is the major mechanism for clearing evoked DA from the extracellular milieu of the NAc (Cass et al., 1993). Nicotine, through the activation of nAChRs, is posited to modulate DAT function thereby contributing to the regulation of synaptic DA concentrations (Hart and Ksir, 1996; Middleton et al., 2004). One possible mechanism is that nicotine-evoked increases in DA release may indirectly lead to an enhancement of the reuptake process as a means to clear the augmented amounts of DA in the synapse. Once DA is released, nicotine may modulate DAT through activation of presynaptic DA D2 receptors or D2 autoreceptors (Hart and Ksir, 1996). This would explain why nicotine-evoked DA release (Fig. 3.8b, 3.9a) and hyperlocomotion (Fig. 3.4) returned to baseline over a shorter time period than bupropion-induced increases in extracellular DA (Fig. 3.8a, b) and locomotor activity (Figs. 3.3, 3.4). Following bupropion-induced inhibition of DAT, elevated levels of DA would be regulated by cell body or terminal D2 receptors. However, due to the absence of DAT function, the time taken for DA clearance would be subsequently increased, thereby prolonging the effects of DA at the synapse. Hence, when administered together, bupropion and nicotine would increase and extend DA-mediated effects as compared to when applied alone, which is consistent with our present findings (Figs. 3.4, 3.8b), but not with those *in vitro* (Chapter 2). This may be explained by the disparities in local tissue concentrations of these drugs *in vitro* and *in vivo*, together with the increased complexity of their neurochemical actions *in vivo* (discussed in Chapter 4, section 4.1). Based on the present findings, it can be speculated that the enhanced effects on dopaminergic function following nicotine and bupropion administration (Figs. 3.4, 3.8b) are a result of additive, as oppose to synergistic, processes. However, when applied locally into the NAc_{core}, the combination of bupropion and nicotine (Fig. 3.9b) failed to enhance the effect of bupropion in the absence of nicotine (Fig. 3.9b). Plausible explanations may be the lack of cell body control on extracellular DA and absence of drug metabolism, since both drugs were applied directly into the NAc_{core}. Another possibility may be that the small increases in extracellular DA following local application of nicotine in the NAc_{core} (Fig. 3.9a), could have been masked by the large within-group variability seen following local application of 100 µM bupropion and nicotine (Fig. 3.9b).

3.4.5 Conclusions

Recent preclinical studies have shown that bupropion may inhibit nAChRs (see section 2.4.2; Fryer and Lukas, 1999; Slemmer et al., 2000; Miller et al., 2002). Furthermore, the actions of bupropion may be concentration-dependent, where

bupropion-induced blockade of DAT occurs at low concentrations of bupropion, whilst at higher concentrations, bupropion-induced inhibition of nAChRs prevails (see section 2.4.4; Rauhut et al., 2003; Bruijnzeel and Markou, 2003). Some of the observations made in the present chapter, particularly the enhancement of nicotine effects (Figs. 3.4, 3.8b), may be due to the weak inhibitory effects on DA reuptake by bupropion. The present *in vivo* experiments focusing on the effects of local application of bupropion and nicotine in the NAc_{core} (Fig. 3.9) were conducted in order to replicate the conditions and concentrations of bupropion used in our previous *in vitro* study (see Chapter 2). However, consistent with a previous study (Shoaib et al., 2003), the present *in vivo* experiments failed to illustrate any nAChR inhibiting property of bupropion (Fig. 3.9b). A likely rationalisation is the complexity in anatomical features, and therefore, the pharmacodynamics of the *in vivo* compared to the *in vitro* model (discussed in Chapter 4, section 4.1).

CHAPTER 4

Integrated Discussion

It is largely accepted that nicotine is one of the, if not the main, active ingredient in tobacco smoke that leads to and maintains tobacco addiction. Its ability to modulate the release of DA in brain areas central to reward mechanisms, has resulted in extensive research efforts dedicated at developing interventions that prevent nicotine dependence (section 1.5). Currently, the most successful pharmacotherapy that facilitates smoking cessation is bupropion. Although the precise mechanism by which bupropion aids smoking cessation remains unclear, there is substantial evidence to implicate dopaminergic, noradrenergic and nicotine antagonistic components in its efficacy as an anti-smoking agent. How these components interact is yet unclear, and is probably very complex. Therefore, the work presented in this thesis has attempted to methodically evaluate nicotine-bupropion interactions, with respect to DA release, in neuronal models of increasing complexity.

4.1 *In vitro* and *in vivo* models of increasing complexity: advantages and limitations

Synaptosomes permit the study of responses by neurotransmitter receptors in isolated nerve terminals, without interference from neurotransmitters released from neighbouring boutons (Chapter 2). This model initiated the idea that bupropion may have a dual action of inhibiting DA reuptake at lower concentrations (0.1-1 μM), and blocking nAChRs at the higher concentration (10 μM). This was proposed from results showing a concentration-dependent decrease in nicotine-evoked [^{3}H]DA release (Fig. 2.8a). Although the interaction of bupropion at DAT was ambiguous due to the continuous removal of released [^{3}H]DA under the conditions of superfusion, which minimised DA reuptake by DAT, bupropion did, however, produce a transient increase in basal release, thereby accentuating some interaction with DAT. The finding that bupropion inhibits nAChRs at the higher concentration is in good agreement with previous work (Miller et al., 2002) and was also supported by the slice model in the present study. Moreover, the selective inhibition of nicotine-evoked increases in Ca^{2+} fluorescence in SH-SY5Y cells (Fig. 2.13a) was consistent with the direct antagonism of nAChRs by bupropion.

Slices allow the study of dynamic interactions between neurotransmitters on neighbouring terminals, which are not evident in synaptosomes due to their spatial

separation, and so represent a higher level of complexity (Chapter 2). This model illustrated a different effect of bupropion to that seen in synaptosomes, by exhibiting an inverted u-shaped concentration-response profile (Fig. 2.8b), as compared to the concentration-related profile seen in synaptosomes. As slices allow for neurotransmitter cross-talk from adjoining terminals, a putative model, which implicated the involvement of inhibitory DA D2 receptors and glutamate release from neighbouring glutamate terminals, was proposed to elucidate the effects of DAT blockade at the lower concentrations of bupropion. The slice preparation also corroborated bupropion-induced inhibition of nAChRs at the higher concentration, consistent with that seen in synaptosomes. Furthermore, data obtained when examining the effects of α-Bgt on nicotine-evoked [^{3}H]DA release were comparable with previous work (Kaiser and Wonnacott, 2000), thereby emphasising the interpretation that α7 nAChRs reside on striatal glutamatergic terminals to indirectly modulate DA release (Kaiser and Wonnacott, 2000). The intricacy of neurotransmitter interactions in slices, as compared to synaptosomes, revealed the kind of effects that the weak DAT blocking property of bupropion may have on overall extracellular levels of [^{3}H]DA following nicotine administration.

The synaptosome model contradicted the well-defined property of nomifensine as a DAT blocker, by illustrating a significant decrease in nicotine-evoked [^{3}H]DA release in its presence (Fig. 2.8a). However, considering the high concentration of nomifensine (10 μM) used in the study and that synaptosomes probably allow only minimal DA reuptake by DAT, it was no surprise that nomifensine had non-specific effects in the synaptosome preparation. In fact, the model indicated that the non-specific effects of 10 μM nomifensine may be attributed to nAChR blockade. This inference was confirmed by nomifensine-induced inhibition of nicotine- (Fig. 2.12a) and KCl-evoked increases in Ca^{2+} fluorescence in SH-SY5Y cells (Fig. 2.12b), indicative of nAChR and VOCC blockade by nomifensine. The same concentration of nomifensine (10 μM) examined in slices, produced effects characteristic of a well-known DAT blocker, by increasing nicotine-evoked [^{3}H]DA release in its presence (Fig. 2.8b). As slices permit greater interaction of nomifensine at DAT, compared to synaptosomes, the potency of 10 μM nomifensine at DAT was proposed to overcome any indirect modulation of [^{3}H]DA release via glutamate release seen in the presence of the lower concentrations of bupropion (0.1-1 μM), thereby producing a net increase in extracellular [^{3}H]DA levels.

The *in vitro* neuronal models revealed that bupropion does in fact interact with DAT and nAChRs and that its effects appear to show differential concentration-dependency. Hence, the *in vitro* examination of bupropion's effects provided a framework for *in vivo* modelling. Microdialysis sampling of extracellular DA provided a more physiological representation for studying nicotine-bupropion interactions and thus increased the level of complexity from the *in vitro* models (Chapter 3). As an index of enhanced limbic dopaminergic function, locomotor activity measures provided a

behavioural correlate to the relative increases in extracellular DA as measured by microdialysis (Chapter 3). Microdialysis experiments showed that although local application of 100 μM bupropion into the NAc_{core} notably increased basal levels of extracellular DA, characteristic of its ability to inhibit DAT, neither 1 μM or 100 μM bupropion significantly affected nicotine-evoked DA release (Fig. 3.9b), unlike that seen in slices at 0.1 μM and 10 μM bupropion. Various factors may account for these differences: (1) the distance for bupropion to diffuse to before reaching brain tissue after passing through the microdialysis probe is likely to be different to that when applied to the slice preparation by superfusion. This disparity will probably result in different local concentrations of bupropion at the brain tissue, depending on the method of application; (2) the duration of bupropion infusion via the microdialysis probes was different to that via superfusion. Also microdialysates were sampled every 20 min, whereas fraction collection in the superfusion experiments were done every 2 min. The difference in temporal resolution may contribute to the varying effects of bupropion on nicotine-evoked DA release; (3) the degree of preservation of brain anatomical integrity when sampling by microdialysis in conscious freely-moving rats is greater than in dissected brain tissue used in the superfusion experiments. This is likely to affect the pharmacodynamics of bupropion and therefore its effects on nicotine-evoked DA release; (4) microdialysis and superfusion experiments examined the effects of bupropion on extracellular DA in the NAc_{core} and striatum, respectively. Although the NAc_{core} is part of the ventral striatum, differences in local circuitry may influence the effects of bupropion on nicotine-evoked DA release; (5) the magnitude of effects, if any, as a result of nicotine-bupropion interactions may have been too low for *in vivo*, as opposed to, *in vitro* detection. Although the increases in basal extracellular DA by bupropion, in striatal slices and following local application into the NAc_{core} via the microdialysis probe, may be credited to its ability to block DAT, bupropion, however, failed to exhibit any nAChR inhibiting property following local application, in combination with nicotine, into the NAc_{core}. Therefore, comparisons between the effects of local application of bupropion on nicotine-evoked DA release as measured by the *in vivo* and *in vitro* paradigms should be made with a degree of caution.

Systemic administration, as opposed to local application of bupropion to the NAc_{core} permits cell body modulation of neuronal firing and neurotransmitter efflux. Furthermore, systemic administration of bupropion enables its concentration to be divided to various target regions throughout the body and so is subject to metabolism and excretion, whereas local application of bupropion into the NAc_{core} significantly evades the pharmacokinetic factors that occur after systemic administration. These factors suggest that systemic administration of bupropion represents a more physiological state than local application, and so is comparable, to a certain degree, to oral delivery of bupropion in humans. When administered systemically, bupropion (30 mg/kg, i.p) at a dose relevant to clinical treatment, enhanced the effects of nicotine-evoked DA release (Fig. 3.8b).

Furthermore, this effect correlated with the enhanced motor activity following systemic bupropion (30 mg/kg, i.p) and nicotine (0.4 mg/kg, s.c), as compared to bupropion (30 mg/kg, i.p) alone (Fig. 3.4). However, these effects were contrary to those seen in the *in vitro* slice experiments. The effects of systemic bupropion on nicotine-evoked DA release *in vivo*, failed to exhibit any indirect modulation of DA release through DAT blockade, as postulated to explain the decreased effect of bupropion (0.1 μM) on nicotine-evoked [^{3}H]DA release. Moreover, the enhanced nicotine-evoked DA release and nicotine-induced hyperlocomotion following bupropion pre-treatment were consistent with an absence of bupropion acting as a nicotinic antagonist. However, as mentioned previously, the effects of systemic bupropion are likely to differ from the local application of bupropion by superfusion.

The present findings from the *in vitro* and *in vivo* experiments, with respect to the effects of bupropion on nicotine-evoked DA release, indicate complex mechanisms of action that depend on the concentration of bupropion and type of model that its effects are examined in. In the *in vitro* models, bupropion exhibited a clear antagonism of nAChRs, in particular at the higher concentration (10 μM). This property of bupropion is consistent with other *in vitro* studies (see section 1.6.2.3; Fryer and Lukas, 1999; Slemmer et al., 2000; Miller et al., 2002). However, its effects as a DAT blocker *in vitro* were somewhat multifaceted. Bupropion's effects in the *in vivo* models were attributed to its ability to block DAT. When administered systemically, alongside nicotine, it seemed to potentiate nicotinic responses. This is comparable with previous studies that have examined its effects in rodent models of nicotine reinforcement (see section 1.6.2.4; Shoaib et al., 2003; Rauhut et al., 2003), where bupropion has shown to increase nicotine self-administration. However, bupropion's ability to inhibit DA reuptake and increase extracellular levels of DA, thereby mimicking, in part, some of the effects of nicotine may be useful in alleviating the negative affective symptoms of nicotine withdrawal, as seen in clinical trials (Shiffman et al., 2000). In support of this hypothesis, bupropion has shown to ameliorate the decreases in brain reward function induced by nicotine withdrawal (see section 1.6.2.5; Cryan et al., 2003a). Furthermore, the fact that bupropion can substitute for nicotine in drug discrimination experiments (see section 1.6.2.6; Wiley et al., 2002; Young and Glennon, 2002; Shoaib et al., 2003) is indicative that it shares nicotine mimicking properties. In the present study, bupropion failed to exhibit any nAChR antagonism *in vivo*. So far, only one study (Slemmer et al., 2000) has shown bupropion to inhibit nAChRs underlying various *in vivo* nicotinic effects (see section 1.6.2.3). However, it must be noted that these effects were observed in mice, which metabolise bupropion to the same extent as humans, whereas rats do not extensively convert bupropion to its metabolites (see section 1.6.1).

4.1.1 Bupropion metabolism and pharmacokinetics

Human studies on the metabolism of bupropion have shown that plasma levels of its major metabolite, hydroxybupropion, are much greater than the concentration of the parent compound (see Table 2a). In mice, hydroxybupropion isomers have proved to possess greater potency in targeting DAT and nAChRs compared to bupropion (Damaj et al., 2004). Moreover, drug discrimination studies in rats have shown that *R,R*-hydroxybupropion attenuates nicotine's effect by as much as 50% (Bondarev et al., 2003), indicating that the metabolite has significant nicotine antagonism activity in an animal model that has previously not shown this effect when examined with bupropion itself (Wiley et al., 2002; Young and Glennon, 2002; Shoaib et al., 2003). It follows that bupropion's antagonism of the effects of nicotine is probably attributable to the active metabolites, and so could possibly be observed in humans. Therefore, a reason why the present study failed to observe any bupropion-induced inhibition of nicotinic responses in the *in vivo* models is probably due to the fact that bupropion's antagonism of nAChRs is likely to be mediated by its active metabolites, which were probably not as abundant as bupropion in the rat plasma or brain, and so were unlikely to block any nAChR-mediated effects.

4.2 Animal models of human cigarette addiction

Studies related to nicotine dependence profit from animal models as they offer the possibility to examine the mechanisms of dependence at a depth not possible with human studies, and to investigate the risk factors for addiction and potential treatment interventions at a preclinical stage. For many years, rat models have routinely been used to replicate various effects that characterise nicotine dependence, such as nicotine reinforcement, as modelled by intravenous nicotine self-administration and conditioned place-preference procedures in rats (Le Foll and Goldberg, 2005). Moreover, as rats are easily bred and produce large litters, they offer the flexibility to conduct a variety of short- or long-term experiments, thereby increasing data productivity. However, as in the present study, the use of rats may have some limitations. For example, we learnt that rats do not metabolise bupropion to the same extent as humans, and so we were unable to observe the nAChR-inhibiting property of bupropion in our *in vivo* models. To overcome this problem, animal models with a bupropion metabolism profile similar to humans (e.g. mice) could be used, or bupropion metabolites, instead of bupropion, could be examined in our rat models. The concept of systematically analysing the interactions of nicotine and

bupropion, with respect to DA release, in simple, robust *in vitro* models to more complex, physiological *in vivo* representations, was an important one. These comparative experiments, which would not have been possible in humans, provided some useful insight into the mechanisms underlying the clinical efficacy of bupropion in smoking cessation and also emphasise the difficulties in trying to identify all the components that contribute to a complex response *in vivo.* However, human studies are ultimately the clinical endpoint in research to develop tools to augment treatment efficacy and so are necessary to validate the animal models.

4.2.1 Is nicotine enough?

Ideally, a successful treatment that aids smoking cessation should attenuate at least the primary reinforcing properties of nicotine, and the pathways that mediate the symptoms of nicotine withdrawal. Along with others, the present study has provided some insight into the acute pharmacological effects of bupropion, which suggest that its clinical success in smoking cessation may be due to the fact that it fulfils the above criteria. However, further clarity into its mechanisms of action need to be addressed in chronic regimes that emulate human intake of the drug during treatment for nicotine dependence. This may be tackled by examining the effects of chronic bupropion and its metabolites in rat and/or mouse models of nicotine dependence and withdrawal. Another approach could be similar to the systematic one illustrated in the present study, by exploring the chronic effects of bupropion and its metabolites on nAChR-evoked neurotransmitter release in neuronal models of increasing complexity. It should be noted that conditioned cues (see section 1.1) paired with the reinforcing properties of nicotine also serve to maintain and reinstate tobacco smoking. Examining the effects of bupropion on the reinstatement of nicotine-seeking behaviour in animal models of nicotine dependence and withdrawal would further our understanding of bupropion's actions as an anti-smoking agent.

4.3 Future perspectives

From the present study, it appears that bupropion is capable of blocking DAT and nAChRs, and that these actions may be separated with respect to bupropion concentration. Bupropion's ability to increase extracellular DA by inhibiting DAT, particularly in the NAc, could reduce the deficit in DA overflow and brain reward function caused by nicotine withdrawal, thereby helping to prevent relapse. Bupropion-induced blockade of nAChRs is likely to be less important at predicted concentrations of bupropion in the brain. However, it is possible that inhibition of nicotinic effects by bupropion may contribute to its ability to prevent relapse in long-term treatment. As longer periods of

nicotine abstinence alone could help break the strength of nicotine reinforcement, bupropion could combine with this effect to help smokers recover from single relapse episodes.

The multifaceted effects that contribute to the clinical efficacy of bupropion in smoking cessation have made it a challenge to design more effective small-molecule therapies, based solely on the knowledge of bupropion's pharmacology. However, its interaction with nAChRs has provided insight into developing agents that can modulate nicotinic effects. Prime candidates are nAChR partial agonists, which can exhibit nicotine-like pharmacology, yet simultaneously display anti-nicotinic effects. A promising candidate is verenicline, an $\alpha 4\beta 2$ nAChR partial agonist, which is presently in Phase III clinical trials as a smoking cessation aid (see section 1.5.2.2). The use of bupropion has also propelled research into other neuropharmacological mechanisms outside of the nicotinic cholinergic system that might lead to effective smoking cessation therapies. Some of the more promising strategies have targeted specific neurotransmitter receptors such as the cannabinoid- or DA D3 receptors, shown to be involved in nicotine dependence (see sections 1.5.2.3 and 1.5.2.4).

In summary, further research exploring the mechanisms owed to the clinical success of bupropion as an anti-smoking agent is clearly required. Using a systematic approach that ranges from simple to more complex animal models that closely resemble human intake of nicotine has the potential to further our understanding of the pharmacological and behavioural effects of nicotine dependence.

APPENDIX

A1 Microdialysis in rats

Microdialysis is an established sampling technique for the *in vivo* measurement of a variety of substances in both blood and tissue. These include neurotransmitters and neuropeptides, enzymes, electrolytes, as well as various hormones and pharmacological agents.

The principle of brain microdialysis is very simple. A physiological medium, closely resembling endogenous cerebrospinal fluid, is applied to a discrete part of the brain via the inlet of a stereotactically implanted probe. The critical part of the probe consists of a semi-permeable membrane and presents a closed perfusion system to the tissue. Once the probe is in place, compounds in the surrounding tissue diffuse down a concentration gradient across the membrane into the perfusate. Continuous removal of this perfusate maintains the concentration gradient. The pore size in the membrane is limited to introduce a cut-off Dalton weight ensuring that the larger molecules such as proteins cannot cross the membrane. This serves to aid perfusate analysis, keeping the perfusate relatively clean due to the exclusion of large molecular weight compounds. The closed system of the probe helps to preserve the integrity of the brain tissue around the probe and avoids continual washing of the tissue that is associated with open-ended perfusion probes.

A1.1 Microdialysis system

Fig. A1 illustrates a typical microdialysis set-up used routinely in laboratories around the world.

With this as background, part of the project involved constructing a microdialysis set-up (Fig. A2) for measuring brain extracellular neurotransmitters, in particular DA, in conscious freely moving rats. This would allow correlations to be made between changes in extracellular DA levels and behaviour.

Fig. A2 illustrates one of six rat microdialysis set-ups constructed with the help of the workshop engineers at the Department of Biology & Biochemistry. The animal enclosure is represented by a large circular plant pot, which has no sharp corners where the tether could catch or tangle due to slack. To it, is attached a counter-balanced lever arm (workshop engineers at the Dept. of Biology & Biochemistry), which minimises stress on the microdialysis probe, by moving vertically and horizontally with the animal to prevent slack in the tether (Fig. A3). Like most lever arms, it uses a mass as the counter-balance.

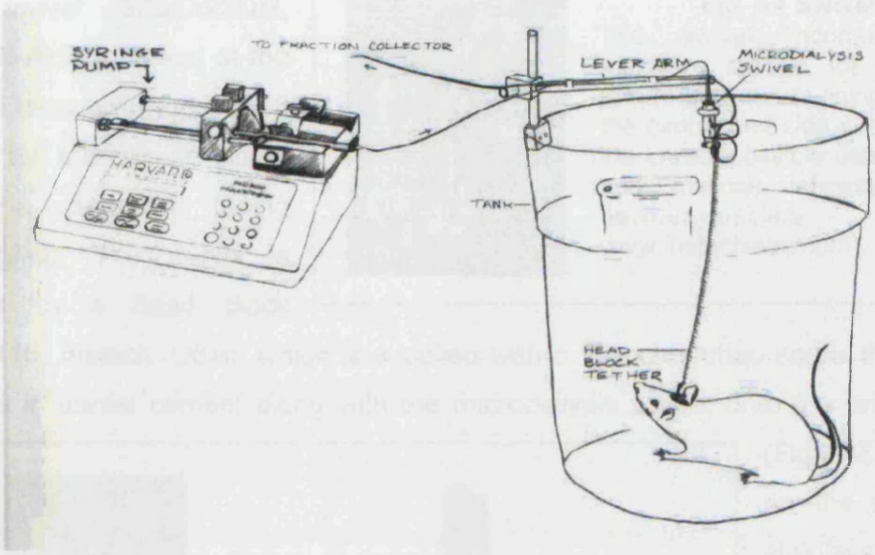


Fig. A1 A standard rat microdialysis system.
This includes an animal enclosure, counter-balanced lever arm, swivel and a head block tether. The apparatus is connected to a swivel pump, via specialised tubing that delivers smooth-flow rates required for microdialysis (source: www.instechlabs.com).

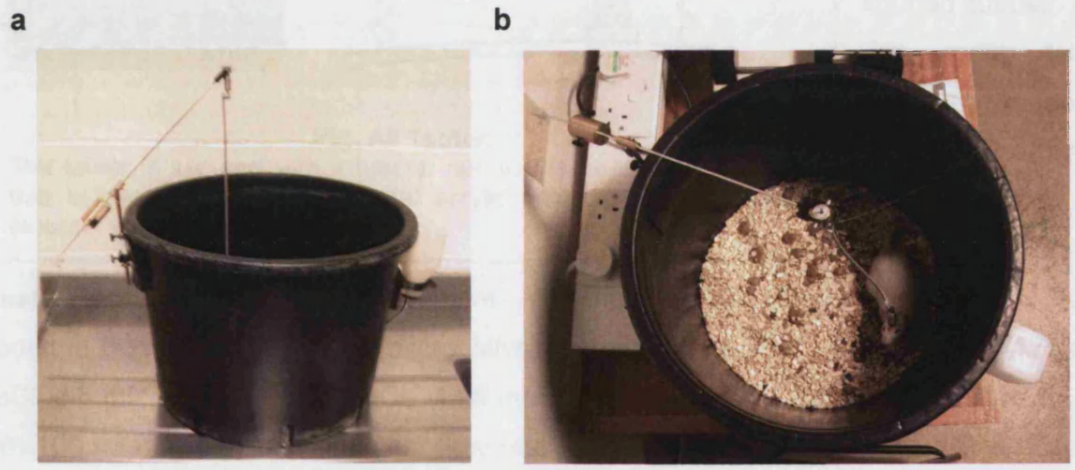


Fig. A2 A rat microdialysis set-up produced at the University of Bath.
(a) and (b) illustrate the front and panoramic view, respectively, of the same components as in Fig. A1.

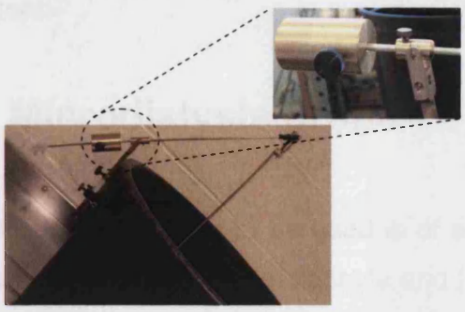


Fig. A3 Counter-balanced lever arm.
Inset shows the mass used for the counter-balance.

A low-torque quartz-lined dual channel swivel (375/D/22QM, Instech, USA) is attached at the end of the lever arm (Fig. A4). It functions by rotating so as to prevent the connecting tubing from tangling. The swivel is connected to a head block tether (M115, Instech, USA), which is supplied with a ¾ inch slotted screw that is partly embedded in dental cement along with the microdialysis probe, onto the animal's skull (Fig. A5). A blade on the end of the stainless steel spring tether slides into the head mount screw and is secured with a knurled tubular nut. The spring tether and the counter-balanced lever arm work together to give the animal greatest possible freedom of movement. A syringe pump (Harvard Apparatus, USA) is placed in close proximity to the microdialysis set-up (Fig. A1). It delivers aCSF (125 mM NaCl, 2.5 mM KCl, 1.18 mM $MgCl_2$, 1.26 mM $CaCl_2$, 2.0 mM Na_2HPO_4, adjusted to pH 7.4 with 100 mM H_3PO_4) at a constant flow rate (1.2 µl/min), via FEP tubing (I.D. 0.12 mm, internal volume 1.2 µl/100 mm length; CMA Microdialysis, Sweden). With excellent resistance to chemicals, ozone, and UV radiation, FEP tubing maintains its chemical resistance. Its non-stick characteristics ease the transfer of product lowering the incidence of protein binding/absorption, making it ideal for the transfer of fluids like neurotransmitters.

Fig. A4 Swivel.
The swivel incorporates a miniature system for ultra-low-torque and quartz lining on both the centre and side channels for the lowest possible dead volume and minimal interaction with neurotransmitters (source: www.instechlabs.com).

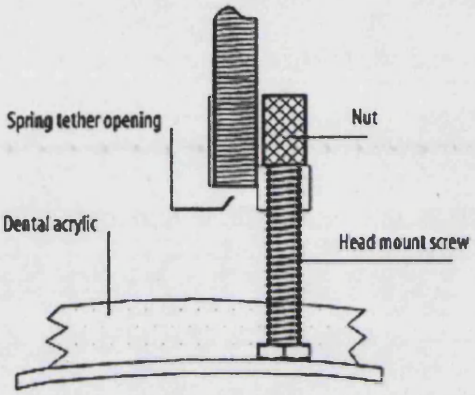


Fig. A5 Tether.
The tether is secured with a tubular nut, to the head mount screw that is partly embedded in dental acrylic onto the animal's skull (source: www.instechlabs.com).

A1.2 Microdialysis probe

The microdialysis probe to be used is of a concentric type. It consists of a semi-permeable dialysis membrane, steel cannula and fused silica tubing. Microdialysis probes

are available commercially, but are expensive. Therefore, homemade concentric probes were produced at minimal costs (Fig. A6b).

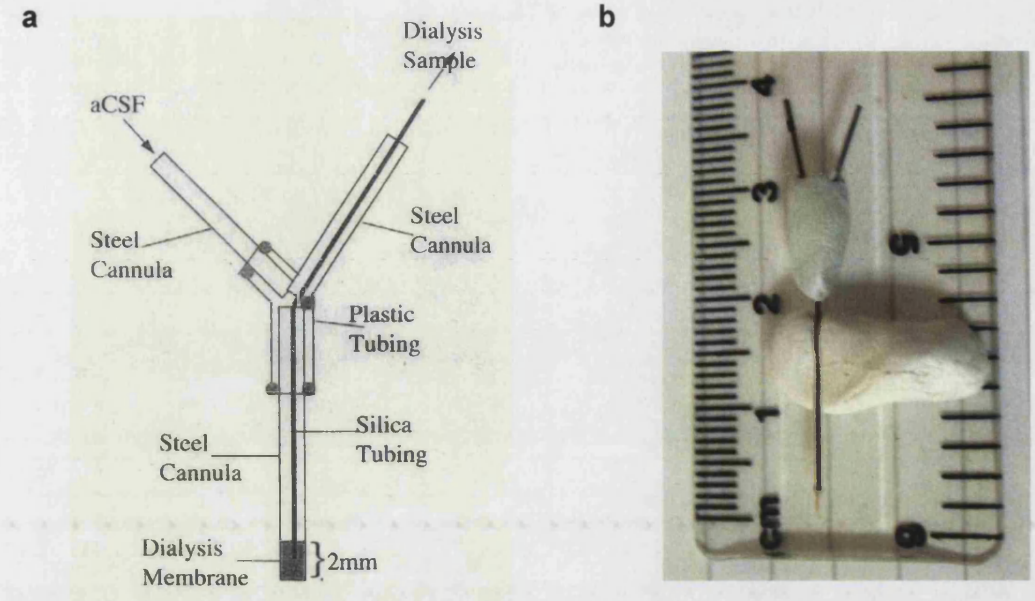


Fig. A6 Microdialysis probe.
(a) Diagrammatic representation of the structure of a concentric microdialysis probe.
(b) Picture of a homemade concentric microdialysis probe. Graduations on the ruler give an indication of the relative size of the probe.

Dialysis tubing (I.D. 0.22 mm, O.D. 0.30 mm, made from polyacrylonitrile with a molecular weight cut-off at 20000 Daltons; Hospal UK Ltd) was obtained from a renal dialysis pack. A 60 mm piece of fine fused silica glass capillary tubing (I.D. 0.11 mm, O.D. 0.17 mm; Scientific Glass Engineering, UK) was threaded into the lumen of a length (~ 50 mm) of dialysis tubing so that it protruded from both ends of the membrane. This supported the membrane during the initial stages of probe preparation. The shaft of the probe consisted of a stainless steel cannula (23 gauge, I.D. 0.33 mm, O.D. 0.60 mm), 22 mm in length (Coopers Needle Works, UK). The dialysis membrane was inserted into one end of the cannula (~ 5 mm) and secured with quickset epoxy resin (RS Components, UK). The resin was allowed to dry for at least 1 h. Once secure, the silica glass was removed and the membrane cut to the desired length (2 mm). A 10 mm length of Portex (polyethylene) tubing (I.D. 0.25 mm, O.D. 0.75 mm) was inserted over the free end of the cannula and a small hole pierced in the tubing, using a 25 gauge needle, close to where it joined the cannula. The silica glass was threaded back into the dialysis tubing, pushed through the steel cannula and out through the hole made in the Portex tubing. The silica glass was pulled through the steel cannula until none was visible inside the membrane, so that the tip of the membrane could be sealed with epoxy resin and left for at least 1 h to dry. After this time, the silica glass was pushed back into the membrane until about 0.5 mm away from the tip of the membrane. The inlet and outlet tubes consisted of stainless

steel cannulas (23 gauge, I.D. 0.33 mm, O.D. 0.60 mm), 11 mm in length (Coopers Needle Works, UK). The inlet was constructed by inserting a cannula into the Portex tubing whilst the outlet was made by placing a cannula over the protruding silica glass. All joints at the base of the probe were sealed with epoxy resin, and once this was dry, all the joints were encased in epoxy putty (RS Components, UK), to strengthen the probe. Care was taken at all stages of preparation not to touch the Hospal membrane in case the probes became blocked.

A1.3 *In vitro* probe recovery

Before the homemade probes were implanted into the rat brains, the recoveries of DA and nicotine across the dialysis membrane were determined *in vitro*. The term recovery or relative recovery is defined as the ratio between the concentration of a particular substance in the perfusate and the concentration of the same substance in the solution outside the probe (Benveniste, 1989).

From every batch of microdialysis probes made, at least four probes were selected for the recovery of DA and nicotine (two probes for each substance). The probes were immersed in a standard solution (of a known concentration, e.g. 50 nM) of either [^{3}H]DA (specificity 43-47 Ci/mmol, 1.67-1.81 TBq/mmol) or (-)-[N-methyl-^{3}H]nicotine ([^{3}H]nicotine; specificity 60 Ci/mmol, 2.2 TBq/mmol, Amersham Biosciences Ltd, Bucks., UK) made up in aCSF. The probes were perfused at a constant flow rate of 1.2 µl/min and allowed to equilibrate for 1 h before samples were taken. Samples were collected every 20 min. Microscint 40 (100 µl) scintillation fluid was added to aliquots (20 µl) of the collected samples and of the standard solutions, and they were counted for radioactivity in a MicroBeta counter (counting efficiency 31%). Recoveries were calculated as a percentage of the radioactivity of substance ([^{3}H]DA or [^{3}H]nicotine) in the sample divided by the radioactivity of substance in the standard solution. Typical recoveries of [^{3}H]DA and [^{3}H]nicotine across the dialysis membrane were between 10-12% for both substances.

Recovery experiments provide valuable information on probe design so that in this way the membrane area can be optimised to give a high recovery value for the substance under investigation. They also serve as a measure of probe to probe variability. Hence, testing for probe recovery was used to determine the reproducibility of the probes and <u>not</u> for correcting results obtained from microdialysis experiments.

A2 High Pressure Liquid Chromatography with Electrochemical Detection

Intracerebral microdialysis is dependent on sensitive analytical methods to measure compounds in perfusate present at very low concentrations (fmol-nmol). HPLC-ECD has provided a tool to investigate the role of neurotransmitters in the central nervous system. The most commonly used separation technique for DA is reverse phase ion-pair chromatography. This combines a column that is packed with C_{18} particles, with a mobile phase consisting of an aqueous buffer, an ion-pair reagent, and an organic solvent.

A successful HPLC system essentially requires the following:

- **Solvent delivery pump:** this pump should have as little pump noise as possible so that the pulsations in the mobile phase are not detected by the electrochemical detection system. Optional use of a pulse dampner may be required to reduce pump noise.

- **Sample injection system:** either a manual-type filled loop valve injectors (Rheodyne) with a 5, 10, 20 or 100 µl loop together with an appropriate syringe (Hamilton), or an appropriate autosampler can be used.

- **Chromatographic columns:** a reverse-phase ion-pair chromatography with a 3 or 5 µm C_{18} silica-based ODS column.

- **Electrochemical detectors:** a detector which uses a high density glassy carbon working electrode, thereby having very low residual current noise resulting in improved chromatographic baselines, and consequent improvement in sensitivity.

- **Recording output from the detector:** usually the detector can be linked to a programmable HPLC integrator or to a suitable computer input.

- **Ancillary equipment:** the mobile phase should be prepared using double

distilled deionised water and needs to be filtered and degassed before use.

Following the construction of a microdialysis set-up, another part of the project involved optimising a HPLC system for the electrochemical detection of DA. This involved determining (1) the optimum voltage at which DA was oxidised and, (2) the system's detection limit for DA.

To determine the optimum voltage for DA oxidation, a known concentration of a DA standard (50 fmol) was injected into the HPLC system, and peak areas were measured at different voltages (Fig. A7). The voltage at which the maximum peak area of DA was obtained, was at 0.60 volts, signifying the optimum voltage for DA oxidation at the electrochemical cell.

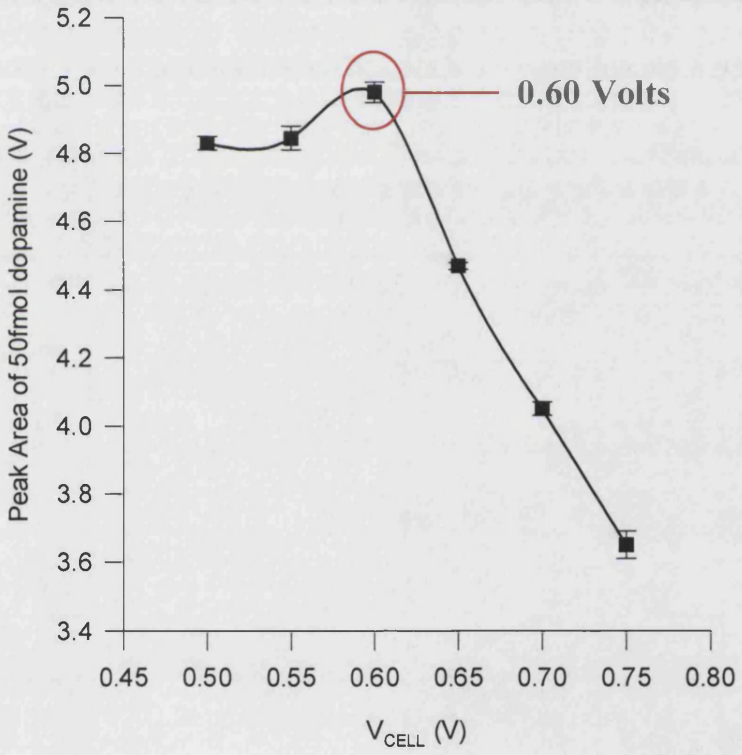


Fig. A7 Voltamogram showing the optimum voltage for DA oxidation.
DA standards (50 fmol) were injected into the HPLC system, and peak areas measured at a range of voltages. Maximum peak area of DA was obtained at 0.60 volts, implicating the optimum voltage of the HPLC system, for DA oxidation. Values are mean ± S.E.M. of duplicate standards.

Next, to determine the detection limit for DA, a range of concentrations of DA standards (1-10 fmol) were injected into the HPLC system, and peak heights were measured at two different sensitivities, 0.2 nA/V and 0.5 nA/V (Fig. A8). The minimum concentration of DA with a peak height that was approximately three fold greater than baseline was accepted as the system's detection limit for DA (Fig. A8a).

Fig. A8a suggests that at a range of 0.5 nA/V, the detection limit for DA was 10 fmol (ratio above baseline = 2.52; ~ 3). However, when the range was decreased to 0.2 nA/V (increased sensitivity), the detection limit for DA was 1 fmol (ratio above baseline = 2.56; ~ 3), indicating a greater sensitivity of the system for the quantification of DA. In confirmation, Fig. A8b indicates that the greater slope at 0.2 nA/V reflects the greater sensitivity of the HPLC system.

a

Dopamine std (fmol)	DA peak height at 0.5nA/V (ratio above baseline)	DA peak height at 0.2nA/V (ratio above baseline)
1	0.46	**2.56**
2	1.03	2.62
3	1.09	4.45
5	1.54	4.64
10	**2.52**	6.96

b

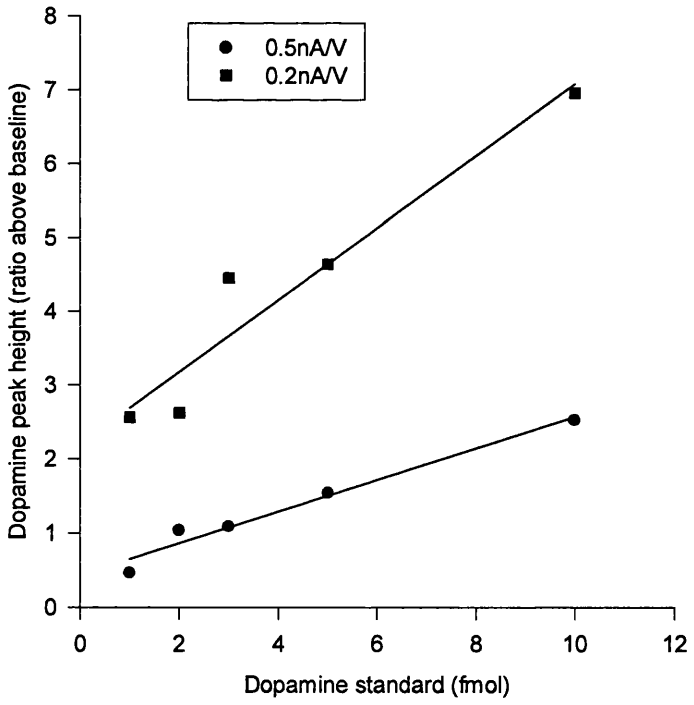


Fig. A8 HPLC system sensitivity results.
(a) Table showing various peak heights of DA standards, expressed as a ratio above baseline. DA standards were measured at two different sensitivities (0.2 and 0.5 nA/V).
(b) The greater slope of 0.2 nA/V reflects the greater sensitivity of the HPLC system.

A3 Final method

To summarise the detection and subsequent quantification of DA using HPLC-ECD (Fig. A9), the method that has been developed in this project employed a Spherisorb

(I.D. 100 x 2.1 mm; Higgins Analytical) reverse-phase column packed with 5 µm C_{18} silica based ODS material. A Bischoff solvent delivery pump with a pulse dampner (PD-120625, Presearch Ltd, Herts, UK) was used to circulate mobile phase (100 mM NaH_2PO_4, 1 mM EDTA, 1 mM octane sulphonic acid, 12% methanol, pH 4.0) at a flow rate of 0.2 ml/min. The mobile phase was filtered through a 0.22 µm filter (Millipore, Bedford, USA) and degassed under vacuum. DA standards (20 µl) were injected onto the column via a refrigerated (4°C) Triathlon autosampler. The standards were prepared daily from a stock solution of DA (1 mM) in a 1:1 mixture of deionised water and 0.1 M perchloric acid, stored at 4°C. Antec-Intro (Leyden, Netherlands) electrochemical detector was used in conjunction with an Antec "wall-jet" design cell (VT-03). The cell employs a high density, glassy carbon working electrode (+0.60 V) combined with an Ag/AgCl reference electrode. The electrode signal was integrated using a PowerChrom data acquisition system (ADInstruments, Oxfordshire, UK).

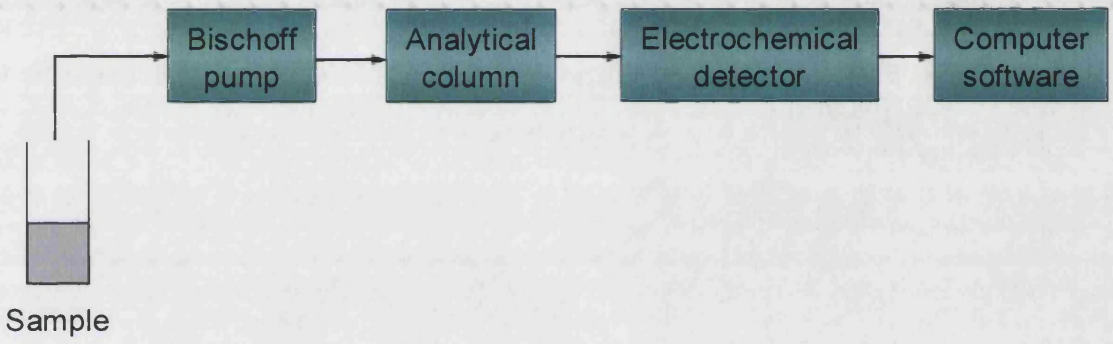


Fig. A9 Flow diagram summarising the basic components and steps required for the functioning of a HPLC system with electrochemical detection.

References

Alex K. D., Yavanian G. J., McFarlane H. G., Pluto C. P., and Pehek E. A. (2005) Modulation of dopamine release by striatal 5-HT2C receptors. *Synapse* **55,** 242-251.

Alkondon M. and Albuquerque E. X. (2005) Nicotinic receptor subtypes in rat hippocampal slices are differentially sensitive to desensitization and early in vivo functional upregulation by nicotine and to block by bupropion. *J Pharmacol Exp Ther.*

Ascher J. A., Cole J. O., Colin J. N., Feighner J. P., Ferris R. M., Fibiger H. C., Golden R. N., Martin P., Potter W. Z., Richelson E., and . (1995) Bupropion: a review of its mechanism of antidepressant activity. *J Clin Psychiatry* **56,** 395-401.

Avshalumov M. V., Chen B. T., Marshall S. P., Pena D. M., and Rice M. E. (2003) Glutamate-dependent inhibition of dopamine release in striatum is mediated by a new diffusible messenger, H2O2. *J Neurosci* **23,** 2744-2750.

Balfour D. J. (2004) The neurobiology of tobacco dependence: a preclinical perspective on the role of the dopamine projections to the nucleus. *Nicotine Tob Res* **6,** 899-912.

Balfour D. J., Benwell M. E., Birrell C. E., Kelly R. J., and Al Aloul M. (1998) Sensitization of the mesoaccumbens dopamine response to nicotine. *Pharmacol Biochem Behav* **59,** 1021-1030.

Balfour D. J., Wright A. E., Benwell M. E., and Birrell C. E. (2000) The putative role of extra-synaptic mesolimbic dopamine in the neurobiology of nicotine dependence. *Behav Brain Res* **113,** 73-83.

Bamford N. S., Zhang H., Schmitz Y., Wu N. P., Cepeda C., Levine M. S., Schmauss C., Zakharenko S. S., Zablow L., and Sulzer D. (2004) Heterosynaptic dopamine neurotransmission selects sets of corticostriatal terminals. *Neuron* **42,** 653-663.

Barik J., Dajas-Bailador F., and Wonnacott S. (2005) Cellular responses to nicotinic receptor activation are decreased after prolonged exposure to galantamine in human neuroblastoma cells. *Br J Pharmacol.*

Belluzzi J. D., Lee A. G., Oliff H. S., and Leslie F. M. (2004) Age-dependent effects of nicotine on locomotor activity and conditioned place preference in rats. *Psychopharmacology (Berl)* **174,** 389-395.

Benowitz N. L. (1996) Pharmacology of nicotine: addiction and therapeutics. *Annu Rev Pharmacol Toxicol* **36,** 597-613.

Benveniste H. (1989) Brain microdialysis. *J Neurochem* **52,** 1667-1679.

Benwell M. E. and Balfour D. J. (1992) The effects of acute and repeated nicotine treatment on nucleus accumbens dopamine and locomotor activity. *Br J Pharmacol* **105,** 849-856.

Benwell M. E. and Balfour D. J. (1997) Regional variation in the effects of nicotine on catecholamine overflow in rat brain. *Eur J Pharmacol* **325,** 13-20.

Berlin I., Aubin H. J., Pedarriosse A. M., Rames A., Lancrenon S., and Lagrue G. (2002) Lazabemide, a selective, reversible monoamine oxidase B inhibitor, as an aid to smoking cessation. *Addiction* **97,** 1347-1354.

Berlin I., Said S., Spreux-Varoquaux O., Launay J. M., Olivares R., Millet V., Lecrubier Y., and Puech A. J. (1995) A reversible monoamine oxidase A inhibitor (moclobemide) facilitates smoking cessation and abstinence in heavy, dependent smokers. *Clin Pharmacol Ther* **58,** 444-452.

Berrettini W. H. and Lerman C. E. (2005) Pharmacotherapy and pharmacogenetics of nicotine dependence. *Am J Psychiatry* **162,** 1441-1451.

Berridge M. J. (1998) Neuronal calcium signaling. *Neuron* **21,** 13-26.

Bondarev M. L., Bondareva T. S., Young R., and Glennon R. A. (2003) Behavioral and biochemical investigations of bupropion metabolites. *Eur J Pharmacol* **474,** 85-93.

Borland L. M. and Michael A. C. (2004) Voltammetric study of the control of striatal dopamine release by glutamate. *J Neurochem* **91,** 220-229.

Bourne J. A. (2003) Intracerebral microdialysis: 30 years as a tool for the neuroscientist. *Clin Exp Pharmacol Physiol* **30,** 16-24.

Boye S. M., Grant R. J., and Clarke P. B. (2001) Disruption of dopaminergic neurotransmission in nucleus accumbens core inhibits the locomotor stimulant effects of nicotine and D-amphetamine in rats. *Neuropharmacology* **40,** 792-805.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72,** 248-254.

Brejc K., van Dijk W. J., Klaassen R. V., Schuurmans M., van Der O. J., Smit A. B., and Sixma T. K. (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **411,** 269-276.

Bressan R. A. and Crippa J. A. (2005) The role of dopamine in reward and pleasure behaviour - review of data from preclinical research. *Acta Psychiatr Scand Suppl* 14-21.

Bruijnzeel A. W. and Markou A. (2003) Characterization of the effects of bupropion on the reinforcing properties of nicotine and food in rats. *Synapse* **50,** 20-28.

Buisson B. and Bertrand D. (2002) Nicotine addiction: the possible role of functional upregulation. *Trends Pharmacol Sci* **23,** 130-136.

Cadoni C. and Di Chiara G. (2000) Differential changes in accumbens shell and core dopamine in behavioral sensitization to nicotine. *Eur J Pharmacol* **387,** R23-R25.

Cao Y. J., Surowy C. S., and Puttfarcken P. S. (2005) Different nicotinic acetylcholine receptor subtypes mediating striatal and prefrontal cortical [(3)H]dopamine release. *Neuropharmacology* **48,** 72-79.

Carr D. B. and Sesack S. R. (2000) Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J Neurosci* **20,** 3864-3873.

Cartier G. E., Yoshikami D., Gray W. R., Luo S., Olivera B. M., and McIntosh J. M. (1996) A new alpha-conotoxin which targets alpha3beta2 nicotinic acetylcholine receptors. *J Biol Chem* **271,** 7522-7528.

Cass W. A., Zahniser N. R., Flach K. A., and Gerhardt G. A. (1993) Clearance of exogenous dopamine in rat dorsal striatum and nucleus accumbens: role of metabolism and effects of locally applied uptake inhibitors. *J Neurochem* **61,** 2269-2278.

Champtiaux N. and Changeux J. P. (2004) Knockout and knockin mice to investigate the role of nicotinic receptors in the central nervous system. *Prog Brain Res* **145,** 235-251.

Champtiaux N., Gotti C., Cordero-Erausquin M., David D. J., Przybylski C., Lena C., Clementi F., Moretti M., Rossi F. M., Le Novere N., McIntosh J. M., Gardier A. M., and Changeux J. P. (2003) Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J Neurosci* **23,** 7820-7829.

Champtiaux N., Han Z. Y., Bessis A., Rossi F. M., Zoli M., Marubio L., McIntosh J. M., and Changeux J. P. (2002) Distribution and pharmacology of alpha 6-containing nicotinic acetylcholine receptors analyzed with mutant mice. *J Neurosci* **22,** 1208-1217.

Changeux J. and Edelstein S. J. (2001) Allosteric mechanisms in normal and pathological nicotinic acetylcholine receptors. *Curr Opin Neurobiol* **11,** 369-377.

Changeux J. P., Bertrand D., Corringer P. J., Dehaene S., Edelstein S., Lena C., Le Novere N., Marubio L., Picciotto M., and Zoli M. (1998) Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. *Brain Res Brain Res Rev* **26,** 198-216.

Chavez-Noriega L. E., Crona J. H., Washburn M. S., Urrutia A., Elliott K. J., and Johnson E. C. (1997) Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h alpha 2 beta 2, h alpha 2 beta 4, h alpha 3 beta 2, h alpha 3 beta 4, h alpha 4 beta 2, h alpha 4 beta 4 and h alpha 7 expressed in Xenopus oocytes. *J Pharmacol Exp Ther* **280,** 346-356.

Cheramy A., Godeheu G., L'Hirondel M., and Glowinski J. (1996) Cooperative contributions of cholinergic and NMDA receptors in the presynaptic control of dopamine release from synaptosomes of the rat striatum. *J Pharmacol Exp Ther* **276,** 616-625.

Clarke P. B., Fu D. S., Jakubovic A., and Fibiger H. C. (1988) Evidence that mesolimbic dopaminergic activation underlies the locomotor stimulant action of nicotine in rats. *J Pharmacol Exp Ther* **246,** 701-708.

Clarke P. B. and Kumar R. (1983) The effects of nicotine on locomotor activity in non-tolerant and tolerant rats. *Br J Pharmacol* **78,** 329-337.

Clarke P. B. and Pert A. (1985) Autoradiographic evidence for nicotine receptors on nigrostriatal and mesolimbic dopaminergic neurons. *Brain Res* **348,** 355-358.

Coe J. W., Brooks P. R., Vetelino M. G., Wirtz M. C., Arnold E. P., Huang J., Sands S. B., Davis T. I., Lebel L. A., Fox C. B., Shrikhande A., Heym J. H., Schaeffer E., Rollema H., Lu Y., Mansbach R. S., Chambers L. K., Rovetti C. C., Schulz D. W., Tingley F. D., III, and O'Neill B. T. (2005) Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation. *J Med Chem* **48,** 3474-3477.

Cohen C., Kodas E., and Griebel G. (2005) CB(1) receptor antagonists for the treatment of nicotine addiction. *Pharmacol Biochem Behav* **81,** 387-395.

Cooper B. R., Hester T. J., and Maxwell R. A. (1980) Behavioral and biochemical effects of the antidepressant bupropion (Wellbutrin): evidence for selective blockade of dopamine uptake in vivo. *J Pharmacol Exp Ther* **215,** 127-134.

Cooper B. R., Wang C. M., Cox R. F., Norton R., Shea V., and Ferris R. M. (1994) Evidence that the acute behavioral and electrophysiological effects of bupropion (Wellbutrin) are mediated by a noradrenergic mechanism. *Neuropsychopharmacology* **11,** 133-141.

Cooper D. C. (2002) The significance of action potential bursting in the brain reward circuit. *Neurochem Int* **41,** 333-340.

Corrigall W. A. and Coen K. M. (1991) Selective dopamine antagonists reduce nicotine self-administration. *Psychopharmacology (Berl)* **104,** 171-176.

Corrigall W. A., Coen K. M., and Adamson K. L. (1994) Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. *Brain Res* **653,** 278-284.

Corrigall W. A., Coen K. M., Zhang J., and Adamson L. (2002) Pharmacological manipulations of the pedunculopontine tegmental nucleus in the rat reduce self-administration of both nicotine and cocaine. *Psychopharmacology (Berl)* **160,** 198-205.

Corrigall W. A., Franklin K. B., Coen K. M., and Clarke P. B. (1992) The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. *Psychopharmacology (Berl)* **107,** 285-289.

Cousins M. S., Stamat H. M., and De Wit H. (2001) Acute doses of d-amphetamine and bupropion increase cigarette smoking. *Psychopharmacology (Berl)* **157,** 243-253.

Covey L. S. (1999) Tobacco cessation among patients with depression. *Prim Care* **26,** 691-706.

Covey L. S., Sullivan M. A., Johnston J. A., Glassman A. H., Robinson M. D., and Adams D. P. (2000) Advances in non-nicotine pharmacotherapy for smoking cessation. *Drugs* **59,** 17-31.

Cryan J. F., Bruijnzeel A. W., Skjei K. L., and Markou A. (2003a) Bupropion enhances brain reward function and reverses the affective and somatic aspects of nicotine withdrawal in the rat. *Psychopharmacology (Berl)* **168,** 347-358.

Cryan J. F., Gasparini F., van Heeke G., and Markou A. (2003b) Non-nicotinic neuropharmacological strategies for nicotine dependence: beyond bupropion. *Drug Discov Today* **8,** 1025-1034.

Cui C., Booker T. K., Allen R. S., Grady S. R., Whiteaker P., Marks M. J., Salminen O., Tritto T., Butt C. M., Allen W. R., Stitzel J. A., McIntosh J. M., Boulter J., Collins A. C., and Heinemann S. F. (2003) The beta3 nicotinic receptor subunit: a component of alpha-conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. *J Neurosci* **23,** 11045-11053.

Dajas-Bailador F. and Wonnacott S. (2004) Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol Sci* **25,** 317-324.

Dajas-Bailador F. A., Heimala K., and Wonnacott S. (2003) The allosteric potentiation of nicotinic acetylcholine receptors by galantamine is transduced into cellular responses in neurons: Ca2+ signals and neurotransmitter release. *Mol Pharmacol* **64,** 1217-1226.

Dajas-Bailador F. A., Mogg A. J., and Wonnacott S. (2002) Intracellular Ca2+ signals evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca2+ channels and Ca2+ stores. *J Neurochem* **81,** 606-614.

Damaj M. I., Carroll F. I., Eaton J. B., Navarro H. A., Blough B. E., Mirza S., Lukas R. J., and Martin B. R. (2004) Enantioselective effects of hydroxy metabolites of bupropion on behavior and on function of monoamine transporters and nicotinic receptors. *Mol Pharmacol* **66,** 675-682.

Dani J. A., Ji D., and Zhou F. M. (2001) Synaptic plasticity and nicotine addiction. *Neuron* **31,** 349-352.

Dani J. A. and Lester R. A. (2001) Nicotinic Acetylcholine Receptors in Neurons. *Encyclopedia of Life Sciences* 1-7.

De Deurwaerdere P., L'hirondel M., Bonhomme N., Lucas G., Cheramy A., and Spampinato U. (1997) Serotonin stimulation of 5-HT4 receptors indirectly enhances in vivo dopamine release in the rat striatum. *J Neurochem* **68,** 195-203.

De Deurwaerdere P., Navailles S., Berg K. A., Clarke W. P., and Spampinato U. (2004) Constitutive activity of the serotonin2C receptor inhibits in vivo dopamine release in the rat striatum and nucleus accumbens. *J Neurosci* **24,** 3235-3241.

Desce J. M., Godeheu G., Galli T., Artaud F., Cheramy A., and Glowinski J. (1992) L-glutamate-evoked release of dopamine from synaptosomes of the rat striatum: involvement of AMPA and N-methyl-D-aspartate receptors. *Neuroscience* **47,** 333-339.

Deutch A. Y. and Cameron D. S. (1992) Pharmacological characterization of dopamine systems in the nucleus accumbens core and shell. *Neuroscience* **46,** 49-56.

Di Chiara G. (1990) In-vivo brain dialysis of neurotransmitters. *Trends Pharmacol Sci* **11,** 116-121.

Di Chiara G. (1998) A motivational learning hypothesis of the role of mesolimbic dopamine in compulsive drug use. *J Psychopharmacol* **12,** 54-67.

Di Chiara G. (1999) Drug addiction as dopamine-dependent associative learning disorder. *Eur J Pharmacol* **375,** 13-30.

Di Chiara G. (2000) Role of dopamine in the behavioural actions of nicotine related to addiction. *Eur J Pharmacol* **393,** 295-314.

Di Chiara G. (2002) Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behav Brain Res* **137,** 75-114.

Di Chiara G. and Imperato A. (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci U S A* **85,** 5274-5278.

Di Matteo V., Pierucci M., and Esposito E. (2004) Selective stimulation of serotonin2c receptors blocks the enhancement of striatal and accumbal dopamine release induced by nicotine administration. *J Neurochem* **89,** 418-429.

Domino E. F. (2001) Nicotine induced behavioral locomotor sensitization. *Prog Neuropsychopharmacol Biol Psychiatry* **25,** 59-71.

Durcan M. J., Deener G., White J., Johnston J. A., Gonzales D., Niaura R., Rigotti N., and Sachs D. P. (2002) The effect of bupropion sustained-release on cigarette craving after smoking cessation. *Clin Ther* **24,** 540-551.

Dwoskin L. P., Teng L., Buxton S. T., Ravard A., Deo N., and Crooks P. A. (1995) Minor alkaloids of tobacco release [3H]dopamine from superfused rat striatal slices. *Eur J Pharmacol* **276,** 195-199.

el Bizri H. and Clarke P. B. (1994) Blockade of nicotinic receptor-mediated release of dopamine from striatal synaptosomes by chlorisondamine and other nicotinic antagonists administered in vitro. *Br J Pharmacol* **111,** 406-413.

Epping-Jordan M. P., Picciotto M. R., Changeux J. P., and Pich E. M. (1999) Assessment of nicotinic acetylcholine receptor subunit contributions to nicotine self-administration in mutant mice. *Psychopharmacology (Berl)* **147,** 25-26.

Epping-Jordan M. P., Watkins S. S., Koob G. F., and Markou A. (1998) Dramatic decreases in brain reward function during nicotine withdrawal. *Nature* **393,** 76-79.

Fagen Z. M., Mansvelder H. D., Keath J. R., and McGehee D. S. (2003) Short- and long-term modulation of synaptic inputs to brain reward areas by nicotine. *Ann N Y Acad Sci* **1003,** 185-195.

Ferrari R., Le Novere N., Picciotto M. R., Changeux J. P., and Zoli M. (2002) Acute and long-term changes in the mesolimbic dopamine pathway after systemic or local single nicotine injections. *Eur J Neurosci* **15,** 1810-1818.

Ferris R. M., Maxwell R. A., Cooper B. R., and Soroko F. E. (1982) Neurochemical and neuropharmacological investigations into the mechanisms of action of bupropion . HCl--a new atypical antidepressant agent. *Adv Biochem Psychopharmacol* **31,** 277-286.

Forster G. L. and Blaha C. D. (2000) Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. *Eur J Neurosci* **12,** 3596-3604.

Forster G. L. and Blaha C. D. (2003) Pedunculopontine tegmental stimulation evokes striatal dopamine efflux by activation of acetylcholine and glutamate receptors in the midbrain and pons of the rat. *Eur J Neurosci* **17,** 751-762.

Foulds J., Burke M., Steinberg M., Williams J. M., and Ziedonis D. M. (2004) Advances in pharmacotherapy for tobacco dependence. *Expert Opin Emerg Drugs* **9,** 39-53.

Fryer J. D. and Lukas R. J. (1999) Noncompetitive functional inhibition at diverse, human nicotinic acetylcholine receptor subtypes by bupropion, phencyclidine, and ibogaine. *J Pharmacol Exp Ther* **288,** 88-92.

Fucile S., Matter J. M., Erkman L., Ragozzino D., Barabino B., Grassi F., Alema S., Ballivet M., and Eusebi F. (1998) The neuronal alpha6 subunit forms functional heteromeric acetylcholine receptors in human transfected cells. *Eur J Neurosci* **10,** 172-178.

Gaddum J. H. (1953) The technique of superfusion. *Br J Pharmacol Chemother* **8,** 321-326.

Garzon M., Vaughan R. A., Uhl G. R., Kuhar M. J., and Pickel V. M. (1999) Cholinergic axon terminals in the ventral tegmental area target a subpopulation of neurons expressing low levels of the dopamine transporter. *J Comp Neurol* **410,** 197-210.

George T. P. and O'Malley S. S. (2004) Current pharmacological treatments for nicotine dependence. *Trends Pharmacol Sci* **25,** 42-48.

Gerdeman G. L., Partridge J. G., Lupica C. R., and Lovinger D. M. (2003) It could be habit forming: drugs of abuse and striatal synaptic plasticity. *Trends Neurosci* **26,** 184-192.

Gerzanich V., Kuryatov A., Anand R., and Lindstrom J. (1997) "Orphan" alpha6 nicotinic AChR subunit can form a functional heteromeric acetylcholine receptor. *Mol Pharmacol* **51,** 320-327.

Giorguieff-Chesselet M. F., Kemel M. L., Wandscheer D., and Glowinski J. (1979) Regulation of dopamine release by presynaptic nicotinic receptors in rat striatal slices: effect of nicotine in a low concentration. *Life Sci* **25,** 1257-1262.

Gonon F. G. (1988) Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. *Neuroscience* **24,** 19-28.

Gotti C. and Clementi F. (2004) Neuronal nicotinic receptors: from structure to pathology. *Prog Neurobiol* **74,** 363-396.

Grady S., Marks M. J., Wonnacott S., and Collins A. C. (1992) Characterization of nicotinic receptor-mediated [3H]dopamine release from synaptosomes prepared from mouse striatum. *J Neurochem* **59,** 848-856.

Grady S. R., Meinerz N. M., Cao J., Reynolds A. M., Picciotto M. R., Changeux J. P., McIntosh J. M., Marks M. J., and Collins A. C. (2001) Nicotinic agonists stimulate acetylcholine release from mouse interpeduncular nucleus: a function mediated by a different nAChR than dopamine release from striatum. *J Neurochem* **76,** 258-268.

Grady S. R., Murphy K. L., Cao J., Marks M. J., McIntosh J. M., and Collins A. C. (2002) Characterization of nicotinic agonist-induced [(3)H]dopamine release from synaptosomes prepared from four mouse brain regions. *J Pharmacol Exp Ther* **301,** 651-660.

Grottick A. J., Trube G., Corrigall W. A., Huwyler J., Malherbe P., Wyler R., and Higgins G. A. (2000) Evidence that nicotinic alpha(7) receptors are not involved in the hyperlocomotor and rewarding effects of nicotine. *J Pharmacol Exp Ther* **294,** 1112-1119.

Hamada M., Higashi H., Nairn A. C., Greengard P., and Nishi A. (2004) Differential regulation of dopamine D1 and D2 signaling by nicotine in neostriatal neurons. *J Neurochem* **90,** 1094-1103.

Hart C. and Ksir C. (1996) Nicotine effects on dopamine clearance in rat nucleus accumbens. *J Neurochem* **66,** 216-221.

Harvey S. C., McIntosh J. M., Cartier G. E., Maddox F. N., and Luetje C. W. (1997) Determinants of specificity for alpha-conotoxin MII on alpha3beta2 neuronal nicotinic receptors. *Mol Pharmacol* **51,** 336-342.

Hayford K. E., Patten C. A., Rummans T. A., Schroeder D. R., Offord K. P., Croghan I. T., Glover E. D., Sachs D. P., and Hurt R. D. (1999) Efficacy of bupropion for smoking cessation in smokers with a former history of major depression or alcoholism. *Br J Psychiatry* **174,** 173-178.

Hays J. T., Hurt R. D., Rigotti N. A., Niaura R., Gonzales D., Durcan M. J., Sachs D. P., Wolter T. D., Buist A. S., Johnston J. A., and White J. D. (2001) Sustained-release bupropion for pharmacologic relapse prevention after smoking cessation. a randomized, controlled trial. *Ann Intern Med* **135,** 423-433.

Heal D. J., Frankland A. T., Gosden J., Hutchins L. J., Prow M. R., Luscombe G. P., and Buckett W. R. (1992) A comparison of the effects of sibutramine hydrochloride, bupropion and

methamphetamine on dopaminergic function: evidence that dopamine is not a pharmacological target for sibutramine. *Psychopharmacology (Berl)* **107,** 303-309.

Heidbreder C. A. and Hagan J. J. (2005) Novel pharmacotherapeutic approaches for the treatment of drug addiction and craving. *Curr Opin Pharmacol* **5,** 107-118.

Henningfield J. E., Stapleton J. M., Benowitz N. L., Grayson R. F., and London E. D. (1993) Higher levels of nicotine in arterial than in venous blood after cigarette smoking. *Drug Alcohol Depend* **33,** 23-29.

Hernandez L. F., Segovia G., and Mora F. (2003) Effects of activation of NMDA and AMPA glutamate receptors on the extracellular concentrations of dopamine, acetylcholine, and GABA in striatum of the awake rat: a microdialysis study. *Neurochem Res* **28,** 1819-1827.

Hogg R. C., Raggenbass M., and Bertrand D. (2003) Nicotinic acetylcholine receptors: from structure to brain function. *Rev Physiol Biochem Pharmacol* **147,** 1-46.

Holm K. J. and Spencer C. M. (2000) Bupropion: a review of its use in the management of smoking cessation. *Drugs* **59,** 1007-1024.

Houghtling R. A., Davila-Garcia M. I., and Kellar K. J. (1995) Characterization of (+/-)(-)[3H]epibatidine binding to nicotinic cholinergic receptors in rat and human brain. *Mol Pharmacol* **48,** 280-287.

Hsyu P. H., Singh A., Giargiari T. D., Dunn J. A., Ascher J. A., and Johnston J. A. (1997) Pharmacokinetics of bupropion and its metabolites in cigarette smokers versus nonsmokers. *J Clin Pharmacol* **37,** 737-743.

Hughes J. R., Stead L. F., and Lancaster T. (2005) Nortriptyline for smoking cessation: A review. *Nicotine Tob Res* **7,** 491-499.

Hurt R. D., Sachs D. P., Glover E. D., Offord K. P., Johnston J. A., Dale L. C., Khayrallah M. A., Schroeder D. R., Glover P. N., Sullivan C. R., Croghan I. T., and Sullivan P. M. (1997) A comparison of sustained-release bupropion and placebo for smoking cessation. *N Engl J Med* **337,** 1195-1202.

Hyman S. E. and Malenka R. C. (2001) Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat Rev Neurosci* **2,** 695-703.

Hyttel J. (1982) Citalopram--pharmacological profile of a specific serotonin uptake inhibitor with antidepressant activity. *Prog Neuropsychopharmacol Biol Psychiatry* **6,** 277-295.

Imperato A., Mulas A., and Di Chiara G. (1986) Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. *Eur J Pharmacol* **132,** 337-338.

Itier V. and Bertrand D. (2001) Neuronal nicotinic receptors: from protein structure to function. *FEBS Lett* **504,** 118-125.

Ito R., Dalley J. W., Howes S. R., Robbins T. W., and Everitt B. J. (2000) Dissociation in conditioned dopamine release in the nucleus accumbens core and shell in response to cocaine cues and during cocaine-seeking behavior in rats. *J Neurosci* **20,** 7489-7495.

Ito R., Robbins T. W., and Everitt B. J. (2004) Differential control over cocaine-seeking behavior by nucleus accumbens core and shell. *Nat Neurosci* **7,** 389-397.

Iyaniwura T. T., Wright A. E., and Balfour D. J. (2001) Evidence that mesoaccumbens dopamine and locomotor responses to nicotine in the rat are influenced by pretreatment dose and strain. *Psychopharmacology (Berl)* **158,** 73-79.

Jensen A. A., Frolund B., Liljefors T., and Krogsgaard-Larsen P. (2005) Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. *J Med Chem* **48,** 4705-4745.

Johnston A. J., Ascher J., Leadbetter R., Schmith V. D., Patel D. K., Durcan M., and Bentley B. (2002) Pharmacokinetic optimisation of sustained-release bupropion for smoking cessation. *Drugs* **62 Suppl 2,** 11-24.

Johnston J. A., Fiedler-Kelly J., Glover E. D., Sachs D. P., Grasela T. H., and DeVeaugh-Geiss J. (2001) Relationship between drug exposure and the efficacy and safety of bupropion sustained release for smoking cessation. *Nicotine Tob Res* **3,** 131-140.

Jones I. W., Bolam J. P., and Wonnacott S. (2001) Presynaptic localisation of the nicotinic acetylcholine receptor beta2 subunit immunoreactivity in rat nigrostriatal dopaminergic neurones. *J Comp Neurol* **439,** 235-247.

Jones I. W. and Wonnacott S. (2004) Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. *J Neurosci* **24,** 11244-11252.

Jorenby D. E., Leischow S. J., Nides M. A., Rennard S. I., Johnston J. A., Hughes A. R., Smith S. S., Muramoto M. L., Daughton D. M., Doan K., Fiore M. C., and Baker T. B. (1999) A controlled trial of sustained-release bupropion, a nicotine patch, or both for smoking cessation. *N Engl J Med* **340,** 685-691.

Kaiser S. and Wonnacott S. (2000) alpha-bungarotoxin-sensitive nicotinic receptors indirectly modulate [(3)H]dopamine release in rat striatal slices via glutamate release. *Mol Pharmacol* **58,** 312-318.

Kaiser S. A., Soliakov L., Harvey S. C., Luetje C. W., and Wonnacott S. (1998) Differential inhibition by alpha-conotoxin-MII of the nicotinic stimulation of [3H]dopamine release from rat striatal synaptosomes and slices. *J Neurochem* **70,** 1069-1076.

Karlin A. (2002) Emerging structure of the nicotinic acetylcholine receptors. *Nat Rev Neurosci* **3,** 102-114.

Kauer J. A. (2004) Learning mechanisms in addiction: synaptic plasticity in the ventral tegmental area as a result of exposure to drugs of abuse. *Annu Rev Physiol* **66,** 447-475.

Kenny P. J. and Markou A. (2001) Neurobiology of the nicotine withdrawal syndrome. *Pharmacol Biochem Behav* **70,** 531-549.

Khiroug S. S., Harkness P. C., Lamb P. W., Sudweeks S. N., Khiroug L., Millar N. S., and Yakel J. L. (2002) Rat nicotinic ACh receptor alpha7 and beta2 subunits co-assemble to form functional heteromeric nicotinic receptor channels. *J Physiol* **540,** 425-434.

Killen J. D., Robinson T. N., Ammerman S., Hayward C., Rogers J., Stone C., Samuels D., Levin S. K., Green S., and Schatzberg A. F. (2004) Randomized clinical trial of the efficacy of bupropion combined with nicotine patch in the treatment of adolescent smokers. *J Consult Clin Psychol* **72,** 729-735.

Klimek V., Zhu M. Y., Dilley G., Konick L., Overholser J. C., Meltzer H. Y., May W. L., Stockmeier C. A., and Ordway G. A. (2001) Effects of long-term cigarette smoking on the human locus coeruleus. *Arch Gen Psychiatry* **58,** 821-827.

Klink R., de Kerchove d. A., Zoli M., and Changeux J. P. (2001) Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *J Neurosci* **21,** 1452-1463.

Koob G. F. (1992) Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci* **13,** 177-184.

Koob G. F., Ahmed S. H., Boutrel B., Chen S. A., Kenny P. J., Markou A., O'Dell L. E., Parsons L. H., and Sanna P. P. (2004) Neurobiological mechanisms in the transition from drug use to drug dependence. *Neurosci Biobehav Rev* **27,** 739-749.

Kosten T. and Owens S. M. (2005) Immunotherapy for the treatment of drug abuse. *Pharmacol Ther.*

Kreek M. J., LaForge K. S., and Butelman E. (2002) Pharmacotherapy of addictions. *Nat Rev Drug Discov* **1,** 710-726.

Kulak J. M., McIntosh J. M., Yoshikami D., and Olivera B. M. (2001) Nicotine-evoked transmitter release from synaptosomes: functional association of specific presynaptic acetylcholine receptors and voltage-gated calcium channels. *J Neurochem* **77,** 1581-1589.

Kulak J. M., Nguyen T. A., Olivera B. M., and McIntosh J. M. (1997) Alpha-conotoxin MII blocks nicotine-stimulated dopamine release in rat striatal synaptosomes. *J Neurosci* **17,** 5263-5270.

Laviolette S. R., Alexson T. O., and van der Kooy D. (2002) Lesions of the tegmental pedunculopontine nucleus block the rewarding effects and reveal the aversive effects of nicotine in the ventral tegmental area. *J Neurosci* **22,** 8653-8660.

Laviolette S. R. and van der Kooy D. (2004) The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci* **5,** 55-65.

Le Foll B., Diaz J., and Sokoloff P. (2003) Increased dopamine D3 receptor expression accompanying behavioral sensitization to nicotine in rats. *Synapse* **47,** 176-183.

Le Foll B. and Goldberg S. R. (2004) Rimonabant, a CB1 antagonist, blocks nicotine-conditioned place preferences. *Neuroreport* **15,** 2139-2143.

Le Foll B. and Goldberg S. R. (2005) Control of the reinforcing effects of nicotine by associated environmental stimuli in animals and humans. *Trends Pharmacol Sci* **26,** 287-293.

Learned-Coughlin S. M., Bergstrom M., Savitcheva I., Ascher J., Schmith V. D., and Langstrom B. (2003) In vivo activity of bupropion at the human dopamine transporter as measured by positron emission tomography. *Biol Psychiatry* **54,** 800-805.

A History of Tobacco (1998) http://www.forces.org/writers/james/files/history.htm

Lei W., Jiao Y., Del Mar N., and Reiner A. (2004) Evidence for differential cortical input to direct pathway versus indirect pathway striatal projection neurons in rats. *J Neurosci* **24,** 8289-8299.

Lukas R. J., Changeux J. P., Le Novere N., Albuquerque E. X., Balfour D. J., Berg D. K., Bertrand D., Chiappinelli V. A., Clarke P. B., Collins A. C., Dani J. A., Grady S. R., Kellar K. J., Lindstrom J. M., Marks M. J., Quik M., Taylor P. W., and Wonnacott S. (1999) International Union of

Pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. *Pharmacol Rev* **51,** 397-401.

Lukas R. J., Norman S. A., and Lucero L. (1993) Characterisation of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y human neuroblastoma clonal line. *Mol Cell Biol* **4,** 1-12.

MacDermott A. B., Role L. W., and Siegelbaum S. A. (1999) Presynaptic ionotropic receptors and the control of transmitter release. *Annu Rev Neurosci* **22,** 443-485.

Madras B. K., Fahey M. A., Bergman J., Canfield D. R., and Spealman R. D. (1989) Effects of cocaine and related drugs in nonhuman primates. I. [3H]cocaine binding sites in caudate-putamen. *J Pharmacol Exp Ther* **251,** 131-141.

Mansvelder H. D., De Rover M., McGehee D. S., and Brussaard A. B. (2003) Cholinergic modulation of dopaminergic reward areas: upstream and downstream targets of nicotine addiction. *Eur J Pharmacol* **480,** 117-123.

Mansvelder H. D., Keath J. R., and McGehee D. S. (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron* **33,** 905-919.

Mansvelder H. D. and McGehee D. S. (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron* **27,** 349-357.

Mansvelder H. D. and McGehee D. S. (2002) Cellular and synaptic mechanisms of nicotine addiction. *J Neurobiol* **53,** 606-617.

Marks M. J., Bullock A. E., and Collins A. C. (1995) Sodium channel blockers partially inhibit nicotine-stimulated 86Rb+ efflux from mouse brain synaptosomes. *J Pharmacol Exp Ther* **274,** 833-841.

Marshall D., Soliakov L., Redfern P., and Wonnacott S. (1996) Tetrodotoxin-sensitivity of nicotine-evoked dopamine release from rat striatum. *Neuropharmacology* **35,** 1531-1536.

Marshall D. L., Redfern P. H., and Wonnacott S. (1997) Presynaptic nicotinic modulation of dopamine release in the three ascending pathways studied by in vivo microdialysis: comparison of naive and chronic nicotine-treated rats. *J Neurochem* **68,** 1511-1519.

Marubio L. M., Gardier A. M., Durier S., David D., Klink R., Arroyo-Jimenez M. M., McIntosh J. M., Rossi F., Champtiaux N., Zoli M., and Changeux J. P. (2003) Effects of nicotine in the dopaminergic system of mice lacking the alpha4 subunit of neuronal nicotinic acetylcholine receptors. *Eur J Neurosci* **17,** 1329-1337.

Mathieu-Kia A. M., Kellogg S. H., Butelman E. R., and Kreek M. J. (2002) Nicotine addiction: insights from recent animal studies. *Psychopharmacology (Berl)* **162,** 102-118.

Maura G., Giardi A., and Raiteri M. (1988) Release-regulating D-2 dopamine receptors are located on striatal glutamatergic nerve terminals. *J Pharmacol Exp Ther* **247,** 680-684.

Meiergerd S. M., Patterson T. A., and Schenk J. O. (1993) D2 receptors may modulate the function of the striatal transporter for dopamine: kinetic evidence from studies in vitro and in vivo. *J Neurochem* **61,** 764-767.

Middleton L. S., Cass W. A., and Dwoskin L. P. (2004) Nicotinic receptor modulation of dopamine transporter function in rat striatum and medial prefrontal cortex. *J Pharmacol Exp Ther* **308,** 367-377.

Mifsud J. C., Hernandez L., and Hoebel B. G. (1989) Nicotine infused into the nucleus accumbens increases synaptic dopamine as measured by in vivo microdialysis. *Brain Res* **478,** 365-367.

Millar N. S. (2003) Assembly and subunit diversity of nicotinic acetylcholine receptors. *Biochem Soc Trans* **31,** 869-874.

Miller D. K., Sumithran S. P., and Dwoskin L. P. (2002) Bupropion inhibits nicotine-evoked [(3)H]overflow from rat striatal slices preloaded with [(3)H]dopamine and from rat hippocampal slices preloaded with [(3)H]norepinephrine. *J Pharmacol Exp Ther* **302,** 1113-1122.

Miyazawa A., Fujiyoshi Y., and Unwin N. (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423,** 949-955.

Nayak S. V., Ronde P., Spier A. D., Lummis S. C., and Nichols R. A. (2000) Nicotinic receptors co-localize with 5-HT(3) serotonin receptors on striatal nerve terminals. *Neuropharmacology* **39,** 2681-2690.

Nelson M. E., Kuryatov A., Choi C. H., Zhou Y., and Lindstrom J. (2003) Alternate stoichiometries of alpha4beta2 nicotinic acetylcholine receptors. *Mol Pharmacol* **63,** 332-341.

NIDA Info Facts - Cigarettes and Other Nicotine Products (2005) National Institute on Drug Abuse, USA.

NIDA Research Report - Nicotine Addiction (1998) National Institute on Drug Abuse, USA.

National Institute on Drug Abuse (2005) www.drugabuse.gov

Nielsen J. A., Shannon N. J., Bero L., and Moore K. E. (1986) Effects of acute and chronic bupropion on locomotor activity and dopaminergic neurons. *Pharmacol Biochem Behav* **24,** 795-799.

Nisell M., Marcus M., Nomikos G. G., and Svensson T. H. (1997) Differential effects of acute and chronic nicotine on dopamine output in the core and shell of the rat nucleus accumbens. *J Neural Transm* **104,** 1-10.

Nisell M., Nomikos G. G., Hertel P., Panagis G., and Svensson T. H. (1996) Condition-independent sensitization of locomotor stimulation and mesocortical dopamine release following chronic nicotine treatment in the rat. *Synapse* **22,** 369-381.

Nisell M., Nomikos G. G., and Svensson T. H. (1994a) Infusion of nicotine in the ventral tegmental area or the nucleus accumbens of the rat differentially affects accumbal dopamine release. *Pharmacol Toxicol* **75,** 348-352.

Nisell M., Nomikos G. G., and Svensson T. H. (1994b) Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. *Synapse* **16,** 36-44.

Nissbrandt H., Elverfors A., and Engberg G. (1994) Pharmacologically induced cessation of burst activity in nigral dopamine neurons: significance for the terminal dopamine efflux. *Synapse* **17,** 217-224.

Nomikos G. G., Damsma G., Wenkstern D., and Fibiger H. C. (1989) Acute effects of bupropion on extracellular dopamine concentrations in rat striatum and nucleus accumbens studied by in vivo microdialysis. *Neuropsychopharmacology* **2,** 273-279.

Nomikos G. G., Damsma G., Wenkstern D., and Fibiger H. C. (1990) In vivo characterization of locally applied dopamine uptake inhibitors by striatal microdialysis. *Synapse* **6,** 106-112.

Nomikos G. G., Damsma G., Wenkstern D., and Fibiger H. C. (1992) Effects of chronic bupropion on interstitial concentrations of dopamine in rat nucleus accumbens and striatum. *Neuropsychopharmacology* **7,** 7-14.

Panagis G., Nisell M., Nomikos G. G., Chergui K., and Svensson T. H. (1996) Nicotine injections into the ventral tegmental area increase locomotion and Fos-like immunoreactivity in the nucleus accumbens of the rat. *Brain Res* **730,** 133-142.

Paxinos G. and Watson C. (1986) The Rat Brain in Stereotaxic Coordinates. Academic Press, New York.

Picciotto M. R., Caldarone B. J., Brunzell D. H., Zachariou V., Stevens T. R., and King S. L. (2001) Neuronal nicotinic acetylcholine receptor subunit knockout mice: physiological and behavioral phenotypes and possible clinical implications. *Pharmacol Ther* **92,** 89-108.

Picciotto M. R., Zoli M., Rimondini R., Lena C., Marubio L. M., Pich E. M., Fuxe K., and Changeux J. P. (1998) Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature* **391,** 173-177.

Pidoplichko V. I., DeBiasi M., Williams J. T., and Dani J. A. (1997) Nicotine activates and desensitizes midbrain dopamine neurons. *Nature* **390,** 401-404.

Pidoplichko V. I., Noguchi J., Areola O. O., Liang Y., Peterson J., Zhang T., and Dani J. A. (2004) Nicotinic cholinergic synaptic mechanisms in the ventral tegmental area contribute to nicotine addiction. *Learn Mem* **11,** 60-69.

Porras G., De Deurwaerdere P., Moison D., and Spampinato U. (2003) Conditional involvement of striatal serotonin3 receptors in the control of in vivo dopamine outflow in the rat striatum. *Eur J Neurosci* **17,** 771-781.

Porras G., Di M., V, De Deurwaerdere P., Esposito E., and Spampinato U. (2002) Central serotonin4 receptors selectively regulate the impulse-dependent exocytosis of dopamine in the rat striatum: in vivo studies with morphine, amphetamine and cocaine. *Neuropharmacology* **43,** 1099-1109.

Prince R. J., Fernandes K. G., Gregory J. C., Martyn I. D., and Lippiello P. M. (1996) Modulation of nicotine-evoked [3H]dopamine release from rat striatal synaptosomes by voltage-sensitive calcium channel ligands. *Biochem Pharmacol* **52,** 613-618.

Puttfarcken P. S., Jacobs I., and Faltynek C. R. (2000) Characterization of nicotinic acetylcholine receptor-mediated [(3)H]-dopamine release from rat cortex and striatum. *Neuropharmacology* **39,** 2673-2680.

Quik M. (2004) Smoking, nicotine and Parkinson's disease. *Trends Neurosci* **27,** 561-568.

Rang H. P., Dale M. M., Ritter J. M., and Moore P. K. (2003) Pharmacology. Churchill Livingstone, London.

Rapier C., Lunt G. G., and Wonnacott S. (1990) Nicotinic modulation of [3H]dopamine release from striatal synaptosomes: pharmacological characterisation. *J Neurochem* **54,** 937-945.

Rauhut A. S., Mullins S. N., Dwoskin L. P., and Bardo M. T. (2002) Reboxetine: attenuation of intravenous nicotine self-administration in rats. *J Pharmacol Exp Ther* **303,** 664-672.

Rauhut A. S., Neugebauer N., Dwoskin L. P., and Bardo M. T. (2003) Effect of bupropion on nicotine self-administration in rats. *Psychopharmacology (Berl)* **169,** 1-9.

Reavill C. and Stolerman I. P. (1990) Locomotor activity in rats after administration of nicotinic agonists intracerebrally. *Br J Pharmacol* **99,** 273-278.

Redolat R., Vidal J., Gomez M. C., and Carrasco M. C. (2005) Effects of acute bupropion administration on locomotor activity in adolescent and adult mice. *Behav Pharmacol* **16,** 59-62.

Richelson E. and Pfenning M. (1984) Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake. *Eur J Pharmacol* **104,** 277-286.

Richmond R. and Zwar N. (2003) Review of bupropion for smoking cessation. *Drug Alcohol Rev* **22,** 203-220.

Ridley D. L., Pakkanen J., and Wonnacott S. (2002) Effects of chronic drug treatments on increases in intracellular calcium mediated by nicotinic acetylcholine receptors in SH-SY5Y cells. *Br J Pharmacol* **135,** 1051-1059.

Ridley D. L., Rogers A., and Wonnacott S. (2001) Differential effects of chronic drug treatment on alpha3* and alpha7 nicotinic receptor binding sites, in hippocampal neurones and SH-SY5Y cells. *Br J Pharmacol* **133,** 1286-1295.

Roberts A. J. and Koob G. F. (1997) The neurobiology of addiction: an overview. *Alcohol Health Res World* **21,** 101-106.

Robinson T. E. and Berridge K. C. (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev* **18,** 247-291.

Role L. W. and Berg D. K. (1996) Nicotinic receptors in the development and modulation of CNS synapses. *Neuron* **16,** 1077-1085.

Rowell P. P. and Li M. (1997) Dose-response relationship for nicotine-induced up-regulation of rat brain nicotinic receptors. *J Neurochem* **68,** 1982-1989.

Rowley H. L., Butler S. A., Prow M. R., Dykes S. G., Aspley S., Kilpatrick I. C., and Heal D. J. (2000) Comparison of the effects of sibutramine and other weight-modifying drugs on extracellular dopamine in the nucleus accumbens of freely moving rats. *Synapse* **38,** 167-176.

Sakai K., Akiyama K., Kashihara K., Tsuchida K., Ujike H., Kuroda S., and Shohmori T. (1997) AMPA receptors modulate dopamine release in the striatum, as measured by brain microdialysis. *Neurochem Int* **30,** 329-336.

Salas R., Cook K. D., Bassetto L., and De Biasi M. (2004) The alpha3 and beta4 nicotinic acetylcholine receptor subunits are necessary for nicotine-induced seizures and hypolocomotion in mice. *Neuropharmacology* **47,** 401-407.

Salin-Pascual R. J., Alcocer-Castillejos N. V., and Alejo-Galarza G. (2003) Nicotine dependence and psychiatric disorders. *Rev Invest Clin* **55,** 677-693.

Salminen O., Murphy K. L., McIntosh J. M., Drago J., Marks M. J., Collins A. C., and Grady S. R. (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol Pharmacol* **65,** 1526-1535.

Schilstrom B., De Villiers S., Malmerfelt A., Svensson T. H., and Nomikos G. G. (2000a) Nicotine-induced Fos expression in the nucleus accumbens and the medial prefrontal cortex of the rat: role of nicotinic and NMDA receptors in the ventral tegmental area. *Synapse* **36,** 314-321.

Schilstrom B., Fagerquist M. V., Zhang X., Hertel P., Panagis G., Nomikos G. G., and Svensson T. H. (2000b) Putative role of presynaptic alpha7* nicotinic receptors in nicotine stimulated increases of extracellular levels of glutamate and aspartate in the ventral tegmental area. *Synapse* **38,** 375-383.

Schilstrom B., Nomikos G. G., Nisell M., Hertel P., and Svensson T. H. (1998a) N-methyl-D-aspartate receptor antagonism in the ventral tegmental area diminishes the systemic nicotine-induced dopamine release in the nucleus accumbens. *Neuroscience* **82,** 781-789.

Schilstrom B., Svensson H. M., Svensson T. H., and Nomikos G. G. (1998b) Nicotine and food induced dopamine release in the nucleus accumbens of the rat: putative role of alpha7 nicotinic receptors in the ventral tegmental area. *Neuroscience* **85,** 1005-1009.

Schmitz Y., Schmauss C., and Sulzer D. (2002) Altered dopamine release and uptake kinetics in mice lacking D2 receptors. *J Neurosci* **22,** 8002-8009.

Schroeder D. H. (1983) Metabolism and kinetics of bupropion. *J Clin Psychiatry* **44,** 79-81.

Seguela P., Wadiche J., Dineley-Miller K., Dani J. A., and Patrick J. W. (1993) Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *J Neurosci* **13,** 596-604.

Semba K. and Fibiger H. C. (1992) Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro- and antero-grade transport and immunohistochemical study. *J Comp Neurol* **323,** 387-410.

Sharples C. G., Kaiser S., Soliakov L., Marks M. J., Collins A. C., Washburn M., Wright E., Spencer J. A., Gallagher T., Whiteaker P., and Wonnacott S. (2000) UB-165: a novel nicotinic agonist with subtype selectivity implicates the alpha4beta2* subtype in the modulation of dopamine release from rat striatal synaptosomes. *J Neurosci* **20,** 2783-2791.

Shiffman S., Johnston J. A., Khayrallah M., Elash C. A., Gwaltney C. J., Paty J. A., Gnys M., Evoniuk G., and DeVeaugh-Geiss J. (2000) The effect of bupropion on nicotine craving and withdrawal. *Psychopharmacology (Berl)* **148,** 33-40.

Shoaib M., Benwell M. E., Akbar M. T., Stolerman I. P., and Balfour D. J. (1994) Behavioural and neurochemical adaptations to nicotine in rats: influence of NMDA antagonists. *Br J Pharmacol* **111,** 1073-1080.

Shoaib M., Sidhpura N., and Shafait S. (2003) Investigating the actions of bupropion on dependence-related effects of nicotine in rats. *Psychopharmacology (Berl)* **165,** 405-412.

Simon J. A., Duncan C., Carmody T. P., and Hudes E. S. (2004) Bupropion for smoking cessation: a randomized trial. *Arch Intern Med* **164,** 1797-1803.

Slemmer J. E., Martin B. R., and Damaj M. I. (2000) Bupropion is a nicotinic antagonist. *J Pharmacol Exp Ther* **295,** 321-327.

Soliakov L., Gallagher T., and Wonnacott S. (1995) Anatoxin-a-evoked [3H]dopamine release from rat striatal synaptosomes. *Neuropharmacology* **34,** 1535-1541.

Soliakov L. and Wonnacott S. (1996) Voltage-sensitive Ca2+ channels involved in nicotinic receptor-mediated [3H]dopamine release from rat striatal synaptosomes. *J Neurochem* **67,** 163-170.

Stahl S. M., Pradko J. F., Haight B. R., Modell J. G., Rockett C. B., and Learned-Coughlin S. (2004) A Review of the Neuropharmacology of Bupropion, a Dual Norepinephrine and Dopamine Reuptake Inhibitor. *Prim Care Companion J Clin Psychiatry* **6,** 159-166.

Stathis M., Scheffel U., Lever S. Z., Boja J. W., Carroll F. I., and Kuhar M. J. (1995) Rate of binding of various inhibitors at the dopamine transporter in vivo. *Psychopharmacology (Berl)* **119,** 376-384.

Stauderman K. A., Mahaffy L. S., Akong M., Velicelebi G., Chavez-Noriega L. E., Crona J. H., Johnson E. C., Elliott K. J., Gillespie A., Reid R. T., Adams P., Harpold M. M., and Corey-Naeve J.

(1998) Characterization of human recombinant neuronal nicotinic acetylcholine receptor subunit combinations alpha2beta4, alpha3beta4 and alpha4beta4 stably expressed in HEK293 cells. *J Pharmacol Exp Ther* **284,** 777-789.

Suckow R. F., Smith T. M., Perumal A. S., and Cooper T. B. (1986) Pharmacokinetics of bupropion and metabolites in plasma and brain of rats, mice, and guinea pigs. *Drug Metab Dispos* **14,** 692-697.

Swan G. E., McAfee T., Curry S. J., Jack L. M., Javitz H., Dacey S., and Bergman K. (2003) Effectiveness of bupropion sustained release for smoking cessation in a health care setting: a randomized trial. *Arch Intern Med* **163,** 2337-2344.

Takahashi A., Camacho P., Lechleiter J. D., and Herman B. (1999) Measurement of intracellular calcium. *Physiol Rev* **79,** 1089-1125.

Tapper A. R., McKinney S. L., Nashmi R., Schwarz J., Deshpande P., Labarca C., Whiteaker P., Marks M. J., Collins A. C., and Lester H. A. (2004) Nicotine activation of alpha4* receptors: sufficient for reward, tolerance, and sensitization. *Science* **306,** 1029-1032.

Tashkin D., Kanner R., Bailey W., Buist S., Anderson P., Nides M., Gonzales D., Dozier G., Patel M. K., and Jamerson B. (2001) Smoking cessation in patients with chronic obstructive pulmonary disease: a double-blind, placebo-controlled, randomised trial. *Lancet* **357,** 1571-1575.

Tatsumi M., Groshan K., Blakely R. D., and Richelson E. (1997) Pharmacological profile of antidepressants and related compounds at human monoamine transporters. *Eur J Pharmacol* **340,** 249-258.

Tella S. R., Ladenheim B., and Cadet J. L. (1997) Differential regulation of dopamine transporter after chronic self-administration of bupropion and nomifensine. *J Pharmacol Exp Ther* **281,** 508-513.

The World Health Report - Making A Difference (1999) World Health Organisation, Geneva.

Tonstad S., Farsang C., Klaene G., Lewis K., Manolis A., Perruchoud A. P., Silagy C., Van Spiegel P. I., Astbury C., Hider A., and Sweet R. (2003) Bupropion SR for smoking cessation in smokers with cardiovascular disease: a multicentre, randomised study. *Eur Heart J* **24,** 946-955.

Toth E., Sershen H., Hashim A., Vizi E. S., and Lajtha A. (1992) Effect of nicotine on extracellular levels of neurotransmitters assessed by microdialysis in various brain regions: role of glutamic acid. *Neurochem Res* **17,** 265-271.

Toth E., Vizi E. S., and Lajtha A. (1993) Effect of nicotine on levels of extracellular amino acids in regions of the rat brain in vivo. *Neuropharmacology* **32,** 827-832.

Turner T. J., Pearce L. B., and Goldin S. M. (1989) A superfusion system designed to measure release of radiolabeled neurotransmitters on a subsecond time scale. *Anal Biochem* **178,** 8-16.

Tutka P., Mosiewicz J., and Wielosz M. (2005) Pharmacokinetics and metabolism of nicotine. *Pharmacol Rep* **57,** 143-153.

Unwin N. (2003) Structure and action of the nicotinic acetylcholine receptor explored by electron microscopy. *FEBS Lett* **555,** 91-95.

Unwin N. (2005) Refined structure of the nicotinic acetylcholine receptor at 4A resolution. *J Mol Biol* **346,** 967-989.

Unwin N., Miyazawa A., Li J., and Fujiyoshi Y. (2002) Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the alpha subunits. *J Mol Biol* **319,** 1165-1176.

Vallejo Y. F., Buisson B., Bertrand D., and Green W. N. (2005) Chronic nicotine exposure upregulates nicotinic receptors by a novel mechanism. *J Neurosci* **25,** 5563-5572.

van den Bree M. B. (2005) Combining research approaches to advance our understanding of drug addiction. *Curr Psychiatry Rep* **7,** 125-132.

Volkow N. D. and Li T. K. (2004) Science and Society: Drug addiction: the neurobiology of behaviour gone awry. *Nat Rev Neurosci* **5,** 963-970.

Voorn P., Vanderschuren L. J., Groenewegen H. J., Robbins T. W., and Pennartz C. M. (2004) Putting a spin on the dorsal-ventral divide of the striatum. *Trends Neurosci* **27,** 468-474.

Wada E., Wada K., Boulter J., Deneris E., Heinemann S., Patrick J., and Swanson L. W. (1989) Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* **284,** 314-335.

Wade J. A., Vaughan P. F., and Peers C. (1998) Hypoxia enhances [3H]noradrenaline release evoked by nicotinic receptor activation from the human neuroblastoma SH-SY5Y. *J Neurochem* **71,** 1482-1489.

Wang J. K. (1991) Presynaptic glutamate receptors modulate dopamine release from striatal synaptosomes. *J Neurochem* **57,** 819-822.

Warner C. and Shoaib M. (2005) How does bupropion work as a smoking cessation aid? *Addict Biol* **10,** 219-231.

Westerink B. H. (1995) Brain microdialysis and its application for the study of animal behaviour. *Behav Brain Res* **70,** 103-124.

Westerink B. H. (2000) Analysis of biogenic amines in microdialysates of the brain. *J Chromatogr B Biomed Sci Appl* **747,** 21-32.

Whiteaker P., Peterson C. G., Xu W., McIntosh J. M., Paylor R., Beaudet A. L., Collins A. C., and Marks M. J. (2002) Involvement of the alpha3 subunit in central nicotinic binding populations. *J Neurosci* **22,** 2522-2529.

Whittaker V. P. (1993) Thirty years of synaptosome research. *J Neurocytol* **22,** 735-742.

Wightman R. M. and Robinson D. L. (2002) Transient changes in mesolimbic dopamine and their association with 'reward'. *J Neurochem* **82,** 721-735.

Wiley J. L., Lavecchia K. L., Martin B. R., and Damaj M. I. (2002) Nicotine-like discriminative stimulus effects of bupropion in rats. *Exp Clin Psychopharmacol* **10,** 129-135.

Wileyto E. P., Patterson F., Niaura R., Epstein L. H., Brown R. A., Audrain-McGovern J., Hawk L. W., Jr., and Lerman C. (2005) Recurrent event analysis of lapse and recovery in a smoking cessation clinical trial using bupropion. *Nicotine Tob Res* **7,** 257-268.

Wise R. A. (2002) Brain reward circuitry: insights from unsensed incentives. *Neuron* **36,** 229-240.

Wise R. A. (2004) Drive, incentive, and reinforcement: the antecedents and consequences of motivation. *Nebr Symp Motiv* **50,** 159-195.

Wonnacott S. (1990) The paradox of nicotinic acetylcholine receptor upregulation by nicotine. *Trends Pharmacol Sci* **11,** 216-219.

Wonnacott S. (1997) Presynaptic nicotinic ACh receptors. *Trends Neurosci* **20,** 92-98.

Wonnacott S., Kaiser S., Mogg A., Soliakov L., and Jones I. W. (2000) Presynaptic nicotinic receptors modulating dopamine release in the rat striatum. *Eur J Pharmacol* **393,** 51-58.

Wonnacott S., Mogg A., Bradley A., and Jones I. W. (2002) Presynaptic nicotinic acetylcholine receptors: subtypes mediating neurotransmitter release, in *Nicotinic Receptors in the Nervous System* (Levin E. D., ed.), pp. 29-49. CRC Press, London.

Wonnacott S., Sidhpura N., and Balfour D. J. (2005) Nicotine: from molecular mechanisms to behaviour. *Curr Opin Pharmacol* **5,** 53-59.

Wooltorton J. R., Pidoplichko V. I., Broide R. S., and Dani J. A. (2003) Differential desensitization and distribution of nicotinic acetylcholine receptor subtypes in midbrain dopamine areas. *J Neurosci* **23,** 3176-3185.

Wu Q., Reith M. E., Walker Q. D., Kuhn C. M., Carroll F. I., and Garris P. A. (2002) Concurrent autoreceptor-mediated control of dopamine release and uptake during neurotransmission: an in vivo voltammetric study. *J Neurosci* **22,** 6272-6281.

Yamamoto B. K. and Davy S. (1992) Dopaminergic modulation of glutamate release in striatum as measured by microdialysis. *J Neurochem* **58,** 1736-1742.

Young R. and Glennon R. A. (2002) Nicotine and bupropion share a similar discriminative stimulus effect. *Eur J Pharmacol* **443,** 113-118.

Zhang H. and Sulzer D. (2003) Glutamate spillover in the striatum depresses dopaminergic transmission by activating group I metabotropic glutamate receptors. *J Neurosci* **23,** 10585-10592.

Zhang M. Y. and Beyer C. E. (2005) Measurement of neurotransmitters from extracellular fluid in brain by in vivo microdialysis and chromatography-mass spectrometry. *J Pharm Biomed Anal.*

Zhou F. M., Liang Y., and Dani J. A. (2001) Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. *Nat Neurosci* **4,** 1224-1229.

Zhou F. M., Wilson C., and Dani J. A. (2003) Muscarinic and nicotinic cholinergic mechanisms in the mesostriatal dopamine systems. *Neuroscientist* **9,** 23-36.

Zhou F. M., Wilson C. J., and Dani J. A. (2002) Cholinergic interneuron characteristics and nicotinic properties in the striatum. *J Neurobiol* **53,** 590-605.

Zoli M., Moretti M., Zanardi A., McIntosh J. M., Clementi F., and Gotti C. (2002) Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. *J Neurosci* **22,** 8785-8789.

"Don't worry. If it turns out tobacco is harmful, we can always give it up."